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Abstract

Title of Dissertation:

Chlamydia muridarum Alters the Immune Environment of the Murine Genital Tract to be More Permissive for Infection with *Neisseria gonorrhoeae* in a Novel Coinfection Model

Rachel A. Vonck, Doctor of Philosophy, 2011

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Coinfection with *Neisseria gonorrhoeae* and *Chlamydia trachomatis* occurs frequently, yet studies of the interaction between these two pathogens within the host have been hindered by the lack of an experimental model. Here we describe development and characterization of a female mouse model of gonococcal and chlamydial coinfection. BALB/c mice were first infected with *C. muridarum*, the mouse chlamydiae, and then inoculated with *N. gonorrhoeae* following estradiol treatment to promote long-term gonococcal colonization. Viable gonococci and chlamydiae were recovered by vaginal swab and organisms were visible in the tissue by immunohistochemical staining. Coinfected mice had a higher percentage of vaginal neutrophils compared to mice infected with either pathogen alone and significantly more gonococci were recovered from coinfected mice compared to mice infected with *N. gonorrhoeae* alone. Increased vaginal concentrations of inflammatory mediators (TNFα

and MIP-2) and down-regulation of antimicrobial peptides genes (CRAMP and SLPI) in *C. muridarum*-infected mice prior to gonococcal challenge led us to hypothesize that chlamydial infection alters the immune environment of the genital tract to be more hospitable for *N. gonorrhoeae*. We screened for changes in gene expression during chlamydial infection using an immune-targeted RT-PCR array. Prior to gonococcal challenge, we observed a two-fold decrease in levels of TLR4 transcript in vaginal material from *C. muridarum*-infected mice compared to uninfected mice, despite increased transcript levels for all other TLRs examined. A significant decrease in the percent of TLR4-positive genital epithelial cells in *C. muridarum*-infected mice compared to uninfected mice was observed by flow cytometry. The biological significance of reduced TLR4 expression was supported by the demonstration that gonococcal colonization was not enhanced during coinfection in TLR4-deficient mice. We conclude that *C. muridarum* infection allows for increased gonococcal colonization by reducing the expression of TLR4 in the genital tract. These results are consistent with recent findings from our laboratory that TLR4 plays a protective role during gonococcal infection and suggest that reduced expression of TLR4 during chlamydial infection may promote infection with other sexually transmitted pathogens. This model of gonococcal and chlamydial coinfection has many potential future applications, including product development for the prevention or treatment of coinfection.

Chlamydia muridarum Alters the Immune Environment of the Murine Genital Tract to be More Permissive for Infection with _Neisseria gonorrhoeae_ in a Novel Coinfection Model

by

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Dedication

To my great-grandmothers, Rachel Liberacki and Esther Van Der Voort, and my grandmothers, Rita Luks and Eleanor Vonck. Because of you, I can do anything.

And to Nate. I miss you.

Table of Contents

Table of Tables

Table of Figures

Chapter 1: Introduction

1.0 Preface

Sexually transmitted infections (STIs) have occurred throughout all of recorded history. The past century has seen significant advances in the treatment of STIs, which has reduced the morbidity and mortality associated with these infections; however, because transmission is inextricably linked to sexual behaviors that are difficult to change, it is unlikely that the problem will ever be eradicated. An important facet of sexually transmitted infections is the disproportionate burden of disease on females and their offspring. Following vaginal intercourse with an infected partner, women are at greater risk than men for acquisition of several STIs, including gonorrhea and chlamydia (82). Additionally, the signs and symptoms of these STIs are usually more common and specific in men, leading to more unrecognized and untreated infections in women. Therefore, despite the availability of effective treatments for many STIs, the consequences of infection on female reproductive health are still a major issue and there is a great need for the development of new strategies for the prevention or treatment of these consequences.

Recent advances in the characterization of the human microbiome have increased awareness of the far-reaching consequences of a polymicrobial environment on the outcome of infection (253). The female genital tract is no exception. The cervix and vagina are colonized with a large number of commensal organisms, which were described by Döderlein in Germany as early as 1894 (73). The complexity of vaginal flora is only just starting to be understood using modern molecular techniques (280).

Thus, in the female genital tract, no single organism exists in isolation. It has been demonstrated that changes in the normal flora of the genital tract can alter host susceptibility to a number of sexually transmitted infections and it is thought that colonization with *Lactobacillus spp.* is protective against genital infection (126, 127, 291, 307). In addition to interaction between commensal and pathogenic organisms in the genital tract, coinfection with multiple pathogenic species is common, particularly with *Neisseria gonorrhoeae* and *Chlamydia trachomatis*, the causative agents of gonorrhea and chlamydia, respectively. An *in vivo* model of gonococcal and chlamydial coinfection is needed to better understand the consequences of coinfection in the context of the complex, polymicrobial environment of the host.

2.0 Gonorrhea and Chlamydia

2.1 Organism Biology

Gonorrhea is caused by the human-specific pathogen *Neisseria gonorrhoeae*. The genus *Neisseria* also includes the pathogenic species *N. meningitidis*, a major cause of bacterial meningitis, and several other non-pathogenic species that are only very rarely associated with disease. *N. gonorrhoeae* was first definitively described by Albert Neisser in 1879, but was not cultured until 1882 by Leistikow and Löffler owing to the fastidious nature of *N. gonorrhoeae*, which requires a complex growth medium (319).

Gonococci are Gram-negative, non-motile, non-encapsulated bacteria that grow in pairs as diplococci. The structure of the gonococcal membrane is typical of Gram-negative bacteria and contains a relatively thin layer of peptidoglycan between the inner and outer membranes (319). Gonococci are not encapsulated like *N. meningitidis*, but

they do frequently release membrane blebs known as outer membrane vesicles (OMVs), which may serve in the delivery of toxic bacterial products to host tissues and as a mechanism for binding and neutralizing host bactericidal factors (254, 255). *N. gonorrhoeae* grown on solid agar has varying colony morphology and colonies take on distinct appearances depending on whether the bacteria are piliated and on which opacity (Opa) proteins are expressed. Piliated colonies are small and convex, while non-piliated colonies are larger and flatter (161). Depending on which Opa proteins are expressed, a colony will range in appearance from transparent to opaque (328, 329, 348). In addition to changes in colony morphology, expression of pili and Opa proteins also alter the ability of *N. gonorrhoeae* to invade host cells (50). Gonococci are primarily extracellular, but can invade host cells by several different mechanisms and are capable of replication inside the host cell (197).

Genital chlamydia, ocular trachoma, and lymphogranuloma venereum (LGV) are caused by *Chlamydia trachomatis*, which like *N. gonorrhoeae*, is a Gram-negative, human-specific pathogen. *C. trachomatis* was first observed by Halberstaedter and Prowazek in conjunctival scrapings from orangutangs that had been inoculated with human trachomatous material in 1907, but it wasn't until the 1930s that the LGV causing organism was grown following intracerebral inoculation of mice and then in eggs. In the 1950s, T'ang isolated the agent of trachoma by inoculating embryonated yolk sacs. In 1965, Gordon and colleagues described growth of chlamydiae in tissue culture, allowing study of the organism in the laboratory (294). The family *Chlamydiaceae* underwent a major taxonomical reclassification in 1999 following the sequencing and phylogenetic comparison of 16S and 23S ribosomal RNA genes from different chlamydial species and

controversially split the family into two genuses (85), which have since been recombined (109). There are two biovars within the species *C. trachomatis*. The LGV biovar consists of four different serovars which cause the sexually transmitted disease lymphogranuloma venerium (LGV). The trachoma biovar contains 14 serovars denoted as A-K, Ba, Da, and Ia. Serovars A-C cause the ocular disease trachoma while the remaining serovars (D-K) are responsible for genital chlamydia (85).

Chlamydiae are obligate intracellular organisms, and thus cannot be grown on solid agar or in liquid culture, but rather within host cells under conditions used for normal cell culture. Chlamydiae exist as either elementary bodies (EBs) or reticulate bodies (RBs). The EB is the non-replicative, metabolically inactive, infectious form of the organism. Upon uptake into the host cell by an active, chlamydiae-induced process (211), the organism inhibits phagolysosomal fusion and resides within the membrane bound chlamydial inclusion (80, 95). Inside the host cell, the EB differentiates into the RB, which is the metabolically active, replicative form of the organism. RBs undergo several rounds of replication within the host cell before differentiation back into the EB state. Infectious particles are released upon host cell lysis in order to initiate subsequent rounds of infection. The length of the chlamydial life cycle varies between serovars and even more between species, but tends to last from 48-72 hours (294).

The cell wall structure of *C. trachomatis* appears to be relatively typical of Gram-negative bacteria, although peptidoglycan has not yet been detected biochemically. However, it is widely accepted in the field that some form of peptidoglycan is present in *Chlamydia* species based on the inhibitory effect of penicillins on growth, the activation of peptidoglycan receptor Nod1 during infection, the presence of the genes involved in

the biosynthetic pathway of peptidoglycan, and the demonstration of enzymatic activity (247). As much as 60% of the outer membrane of *Chlamydia* consists of the major outer membrane protein (OmpA or MOMP) (41) and disulfide cross-linking of OmpA with itself and other cysteine rich outer membrane proteins is responsible for much of the structural rigidity of the organism (117, 224). The EB form is compact and electron dense with a diameter of only 350 nm while the RB is less dense and much larger in size at 1 μm in diameter (294). EBs have considerably greater levels of disulfide bonding in the outer membrane allowing for structural rigidity and the ability to exist in the extracellular environment, while the less disulfide bonded RB is not stable outside of the host cell (223).

2.2 Clinical Manifestations and Disease

Gonorrhea and chlamydia have very similar disease manifestations and symptomology. In men, urethritis is the most common manifestation of uncomplicated gonococcal or chlamydial infection and it is difficult to reliably distinguish the mucopurulent discharge of gonorrhea from other non-gonococcal causes of urethritis, including chlamydia. While cervicitis is the most common manifestation of gonococcal or chlamydial infection in women of child-bearing age, it is estimated that approximately 70-90% of women with gonococcal cervicitis also have urethral colonization with *N. gonorrhoeae* (13, 295, 332). Co-colonization of the cervix and urethra with *C. trachomatis* appears to occur at a slightly lower frequency, with estimates ranging from 50-75% (35, 233, 235, 325). For both infections, the symptoms of cervicitis vary and can include vaginal discharge, dysuria, intermenstrual bleeding and menorrhagia, and the

cervix may appear abnormal with the presence of a mucopurulent discharge, erythema and edema, and mucosal bleeding (136, 321).

Gonococcal and chlamydial infections can ascend to the upper reproductive tract (URT) of both men and women. In men, URT infection generally causes epididymitis and acute or chronic prostatitis with more severe complications, such as infertility, occurring only very rarely (82, 136, 321). The consequences of ascendant infection in women tend to be more common and more severe (82). Gonococcal and chlamydial infections of the female URT result in acute salpingitis or pelvic inflammatory disease (PID), the complications of which include chronic pelvic pain, ectopic pregnancy, and infertility. PID is estimated to occur in approximately 10-45% of females with gonorrhea (84, 135, 258) and 10-30% of females with chlamydia (322, 356). Of women with PID, approximately 25% suffer from long-term sequelae, with 20% experiencing chronic pelvic pain, 20% becoming infertile, and 10% developing an ectopic pregnancy, a life-threatening situation for both the mother and the fetus (43, 355).

In a small percentage (0.5-3%) of untreated cases of mucosal gonorrhea, infection disseminates into the bloodstream causing disseminated gonococcal infection (DGI) (134, 186, 228). DGI is more common in women than in men and generally manifests as an arthritis/dermatitis syndrome (115, 186). Chlamydial infection can also disseminate into the bloodstream and to the joints (158) and is associated with the development of Reiter's syndrome, which is characterized by urethritis, conjunctivitis, and arthritis, or reactive arthritis without other symptoms (106, 321). Reiter's syndrome occurs more frequently in men than in women (16) and is associated with certain HLA haplotypes (28, 159).

While the reproductive tract is the most common site of gonococcal and chlamydial infections, several other mucosal sites can also be affected. Rectal, pharyngeal, and ocular infection with either pathogen can occur. While the pharynges are not usually tested for gonococcal infection, it is estimated that 3-7% of heterosexual men, 10-20% of heterosexual females, and 10-25% of homosexual men with genital infection also have pharyngeal infection (29, 167, 333, 358). Chlamydial infection of the pharynges is thought to be less common and in a single study among patients with confirmed genital chlamydial infections, less than 3% of men and 7% of women were also colonized in the pharynges (359)

Both *N. gonorrhoeae* and *C. trachomatis* can be passed to a neonate from an infected mother during delivery and due to the potential for severe disease upon neonatal infection, screening of pregnant women for gonorrhea and chlamydia is especially important (366). *N. gonorrhoeae* and *C. trachomatis* can both cause opthalmia neonatorum, which is a severe conjunctival disease that may result in blindness if not treated properly. *C. trachomatis* infection can also manifest as pneumonia in the newborn at 1-3 months of age. Several other body sites can be infected in the neonate during birth, including the urogenital tract and oropharynx, and infection can disseminate into the bloodstream resulting in arthritis or meningitis in the infant (136, 321, 366).

2.3 Epidemiology

Chlamydia and gonorrhea are consistently the first and second most common reportable infections in the United States, respectively (46). In 2009, over 1.2 million cases of chlamydia were reported to the Centers for Disease Control, up 2.8% over the number reported in 2008 (46). There are approximately three times as many women with

chlamydia than men and incidence is highest in the 15-19 year old age group (46). Due to high rates of asymptomatic chlamydial infection, it is estimated that in the U.S. approximately 3 million infections actually occur per year (353). In contrast, many fewer cases of gonorrhea were reported to the CDC in 2009 at 301,174 cases (46). Asymptomatic gonococcal infections are also common, however, and estimates of actual case rates in the U.S. annually are around 700,000 (353). Following a dramatic 75% decrease in gonorrhea from 1975 to 1997, levels in the United States held at a stable plateau until 2006 (44). From 2006 to 2009 there was a steady decline in the incidence of gonorrhea, including a 10% decrease from 2008 to 2009 (46). Despite the relatively stable incidence of gonorrhea, the age group most commonly afflicted has changed over the past few years with a decrease in the number of cases seen in the 25-29 year old age group and a concurrent increase in the 15-19 year old age group (44). Unlike chlamydia, cases of gonorrhea reported in men and women are approximately equal (46).

2.4 Incidence of Gonococcal and Chlamydial Coinfection

It is well-understood that genital coinfection with *N. gonorrhoeae* and *C. trachomatis* is common (Figure 1) and current CDC Guidelines for the Treatment of Sexually Transmitted Diseases recommend treatment of gonorrhea with a regimen that is also effective against chlamydia (366). Specific estimates of coinfection rates tend to vary for several reasons, including differences in detection methods, sample size, and the characteristics of the population studied (227). For example, estimates tend to increase in a population that self-selects for symptoms by visiting an STD or family planning clinic as compared to a random sampling from an unselected population. For individuals with gonorrhea, rates of chlamydial coinfection range from 20-70% in women (23, 38, 49, 61,

68, 72, 75, 94, 101, 125, 155, 182, 199, 227, 371) and 3-70% in men (61, 68, 72, 125, 155, 182, 199, 227, 288). For individuals with chlamydia, rates of gonococcal coinfection range from 4-30% in women (23, 38, 61, 94, 101, 155, 199, 371) and 8-30% in men (61, 155, 199, 227).

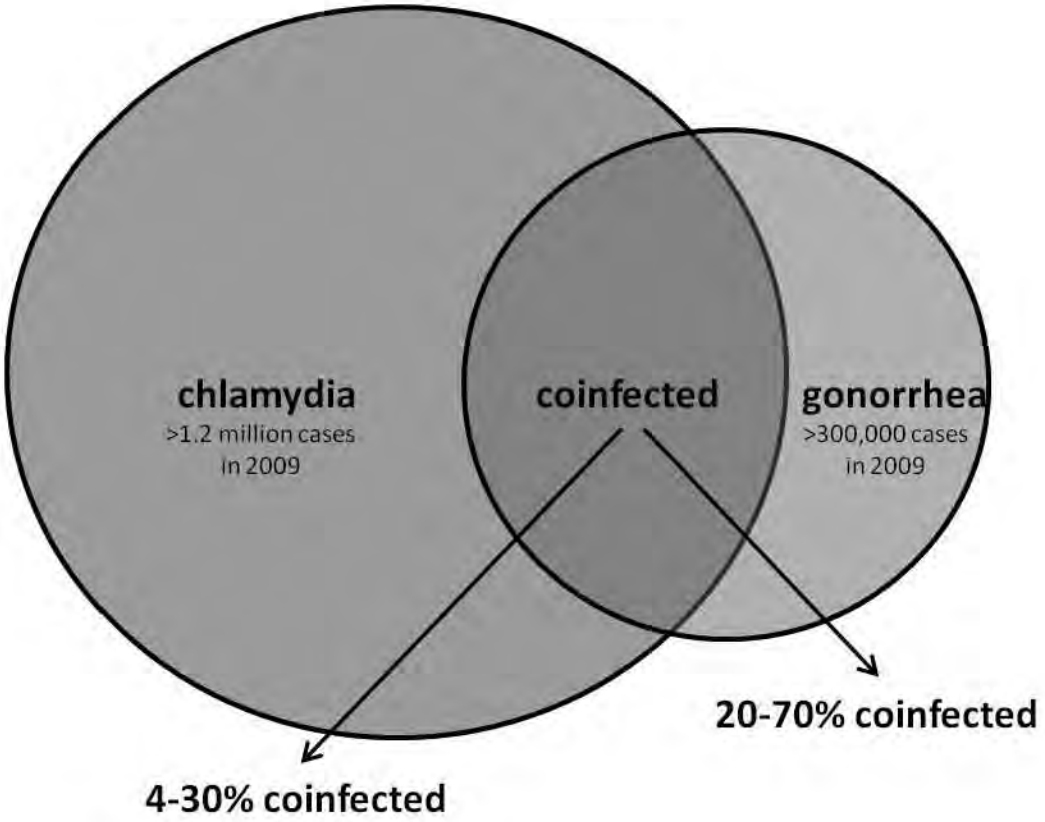
It is not known whether the generally lower rate of gonococcal coinfection in patients with chlamydia and higher rate of chlamydial coinfection in patients with gonorrhea is a result of the greater overall incidence of chlamydia in most populations (46) or due to other biological factors. Additionally, due to high rates of asymptomatic infection with both *N. gonorrhoeae* and *C. trachomatis*, little is known about coinfection outside of the health care setting. Two studies were published in 2004 in which incidence of coinfection was assessed in adolescent and young adult populations in the United States without selection through the health care system. Nsuami and colleagues performed school-based screening during the 1998-1999 school year in an urban high school, including approximately 6000 students ranging in age from 14 to 20 years old. In this study, approximately 43% of students with gonorrhea were coinfected with *C. trachomatis* and approximately 11% of students with chlamydia were coinfected with *N. gonorrhoeae* with little difference in rates by gender (227). Miller and colleagues screened young adults ages 18-26 from across the United States in 2001 and 2002 and found a higher rate (70%) of chlamydial coinfection in individuals infected with *N. gonorrhoeae*, and slightly lower rate (7.5%) of gonococcal coinfection in individuals infected with *C. trachomatis*, but similarly saw little to no difference in rates of coinfection by gender (199). Regardless of the exact frequency, it is clear that in men

and women 25 years and younger in the United States, coinfection with *N. gonorrhoeae* and *C. trachomatis* is a major concern (366).

Figure 1. Incidence of gonococcal and chlamydial coinfection.

In 2009, over 1.2 million cases of chlamydia and 300,000 cases of gonorrhea were reported to the Centers for Disease Control (353). Estimates of coinfection vary and ranges based on available studies are shown here. It is estimated that 4-30% of individuals with chlamydia are coinfected with *N. gonorrhoeae* and that 20-70% of individuals with gonorrhea are coinfected with *C. trachomatis* (23, 38, 49, 61, 68, 72, 75, 94, 101, 125, 155, 182, 199, 227, 288, 371).

Figure 1. Incidence of gonococcal and chlamydial coinfection.



2.5 Laboratory Diagnosis and Treatment

In December of 2010, the Centers for Disease Control and Prevention issued Sexually Transmitted Disease Treatment Guidelines (366), which detail the most current recommended diagnostic methods, treatment regimens, and follow-up protocols for patients with STIs. A description of the CDC recommendations for the diagnosis and treatment of gonorrhea and chlamydia follows.

Annual screening for infection with *N. gonorrhoeae* and *C. trachomatis* is recommended for all women ≤ 25 years of age and older women with risk factors such as new or multiple sex partners. Routine screening of males is not recommended, except in settings with high prevalence such as STD clinics and correctional facilities. Screening is especially important in pregnant women in order to prevent severe disease in the newborn. Nucleic acid amplification tests (NAATs) are most frequently used to diagnose both gonococcal and chlamydial infections using urine or endocervical specimens, although culture and other detection methods are still used infrequently. In the case of diagnosis with either gonorrhea or chlamydia, patients should be tested for other STIs.

Prompt treatment of infection with either *N. gonorrhoeae* or *C. trachomatis* is important, especially in women due to the potential development of PID and its complications and to prevent transmission to sexual partners. The recommended treatment for chlamydia is azithromycin (1 g orally in a single dose) or doxycycline (100 mg orally twice daily for 7 days). While both treatments are equally effective with a 97-98% cure rate, if patient compliance is questionable the single dose of azithromycin is recommended. Due to increases in the resistance of *N. gonorrhoeae* to penicillins, tetracyclines, older macrolides such as erythromycin and increasingly to azithromycin,

only cephalosporins are recommended for use in the treatment of gonorrhea in the United States. However, several cases of infection with cephalosporin resistant strains of *N. gonorrhoeae* have been reported in Asia. Additionally, due to high rates of coinfection with *C. trachomatis* in individuals infected with *N. gonorrhoeae*, presumptive treatment for chlamydia is recommended upon diagnosis with gonorrhea at any mucosal site. Thus, the recommended treatment regimen for gonorrhea includes a single intramuscular injection of 250 mg ceftriaxone plus treatment for chlamydia with either azithromycin or doxycycline. Azithromycin alone is insufficient for the treatment of gonococcal and chlamydial coinfection due to the aforementioned resistance of *N. gonorrhoeae* to this antibiotic.

Following antibiotic treatment, test-of-cure is not recommended for infection with either *N. gonorrhoeae* or *C. trachomatis* unless symptoms persist. However, reinfection with both pathogens is quite common, in large part due to failure of an infected partner to be treated, and repeat testing is recommended after 3 months for both males and females in order to assess reinfection. Testing for reinfection is especially important in women as the risk for PID increases upon repeat infection.

3.0 Pathogenesis and Host Response

3.1 Gonococcal Virulence Factors

N. gonorrhoeae is a highly host-adapted pathogen and has over time evolved many mechanisms for the evasion of host defenses. The gonococcus has both phase and antigenically variable proteins on its outer membrane, which results in a constantly changing bacterial surface and a diverse population of bacteria at the site of infection.

Gonococci can also directly and indirectly inhibit or block the function of innate and adaptive immune effectors and some gonococcal products have direct toxicity to human tissue (Figure 2).

Many gonococcal virulence factors undergo phase variation, including opacity (Opa) proteins and lipooligosaccharide (LOS), which results in populations of bacteria that either do or do not express these factors. Opa proteins are known to be involved in adherence and invasion and likely have other functions, such as immune evasion (119). Each gonococcal strain has genes for 11-12 Opa proteins and can express as many as 4-5 Opa proteins at one time (24). Individual Opa proteins may be antigenically distinct due to sequence differences in the hypervariable region of the protein (14). Phase variation occurs via a frameshift mechanism that results in variation in the number of pentameric repeats downstream of the start codon, which determines whether the transcript will be in frame for translation (219). Selection for Opa-expressing variants is seen during experimental infection of male volunteers (145) and Opa-expression phenotype varies over the course of the reproductive cycle in women (143) and female mice (56, 311), suggesting an important role for Opa proteins during gonococcal infection.

The mechanism by which gonococcal LOS undergoes phase variation is similar to the mechanism of *opa* gene phase variation. The LOS of *N. gonorrhoeae* is shorter than traditional LPS because it lacks the polymeric sugar attached to the KDO containing core polysaccharide moiety. Each gonococcal strain can make up to 6 forms of LOS, which vary in their core sugar antigens. Antigenic variation of LOS is caused by reversible frame shift mutations in several genes involved in the synthesis pathway. Variation in LOS alters the ability of the gonococcus to be sialylated through the addition of host

neuraminic acid to LOS on the surface of the gonococcus by sialyltransferase (261). Sialylation of LOS containing a lacto-N-neotetraose moiety confers what is defined as unstable serum resistance to gonococcal isolates because it is dependent on phase variable LOS. Shorter moieties of LOS cannot be sialylated and variants expressing these LOS types tend to be serum sensitive and more invasive than variants expressing longer LOS moieties (107, 242, 313, 339).

Like Opa proteins and LOS, the type IV colonization pili of *N. gonorrhoeae* are also phase variable, however, antigenic variation of pili also occurs via a mechanism that is independent of phase variation. Because most human isolates are piliated (160), it is the antigenic variation of pili that is likely most important during infection. Adherence to mucosal epithelial cells is initiated by colonization pili (249), which consist primarily of pilin subunits PilE (301). One or two expressed *pilE* genes and 6-8 silent loci exist on the chromosome of *N. gonorrhoeae*. Recombination events between pilin loci result in the movement of pilin sequence from a silent locus to an expression locus, which causes the expression of antigenically different pilin (320). The potential for many different recombination events among *pilE* loci results in a nearly limitless repertoire of antigenic types.

Many antimicrobial innate immune effectors are present at the genital mucosa, including complement, antimicrobial peptides, and reactive oxygen species (ROS), and the gonococcus has several redundant means to evade these defenses. As mentioned previously, sialylation of LOS results in unstable serum resistance by inhibiting complement deposition (313). The gonococcal outer membrane porin mediates stable serum resistance because it is consitutively expressed. Porin exists as two classes, PIA

and PIB, and a given strain has only one allele. PIA strains are more serum resistant due to the ability of porin to bind complement regulatory proteins factor H (fH) and C4 binding protein (C4BP), which inhibits complement deposition on the surface of the bacterium. The porin of PIB strains binds only C4BP, not fH (266, 354). Antimicrobial peptides can be actively expelled from the gonococcus. *N. gonorrhoeae* expresses several efflux pumps, including the MtrCDE system, which expels a wide variety of hydrophobic agents including detergents, antibiotics, and antimicrobial peptides (112, 309). The MtrCDE efflux pump is under the control of both a repressor (MtrR) and an activator (MtrA) (113). Low levels of substrate trigger up-regulation of pump expression and, in a murine model, de-repression of the pump allows for increased fitness, even in the absence of antibiotic treatment, suggesting that natural substrates like antimicrobial peptides challenge the gonococcus *in vivo* (350, 351). *N. gonorrhoeae* also has several redundant antioxidant systems for defense against oxidative stress, including the MntABC manganese transporter, catalase, cytochrome *c* peroxidase, and methionine sulfoxide reductase (MsrA) (304). The role of these antioxidants, however, is not entirely clear. While hydrogen peroxide-producing lactobacilli can inhibit gonococcal growth *in vitro*, there is no effect *in vivo* (214). Similarly, gonococci are able to survive and replicate within PMNs (312), but no single antioxidant enzyme appears to be required for survival (303). However, mutants in *N. gonorrhoeae* that were more sensitive to *in vitro* killing by ROS were also less able to survive in primary human cervical epithelial cells, suggesting a role for gonococcal antioxidant defenses in intraepithelial survival (305).

In addition to the ability to evade innate immune effectors, the gonococcus also has several means for evasion of the adaptive immune response, in particular the

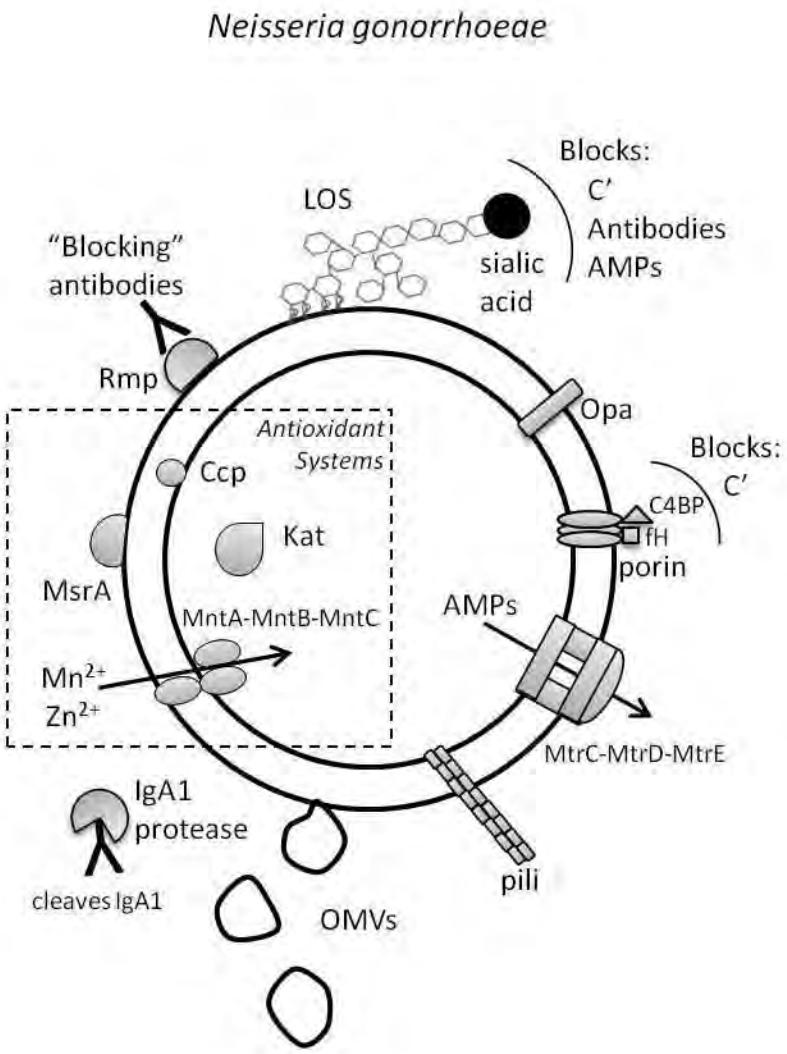
avoidance of antibody-mediated killing. As with complement, sialylation of LOS also inhibits the binding of antibodies to the surface of the organism, preventing antibody-mediated bacteriolysis (70). Gonococci produce immunoglobulin A (IgA1) protease, which cleaves and inactivates mucosal IgA1 (216). IgA1 protease is also thought to allow for intracellular growth of the organism by allowing for phagosomal compartmentalization (118). The ability of gonococci to invade and survive within host cells is another mechanism of avoiding antibodies and other mucosal immune factors, such as complement. Additionally, it is thought that "blocking antibodies" generated against the outer membrane reduction modifiable protein (Rmp) bind to the surface of the bacterium, preventing protective antibodies from binding (217, 259).

Finally, although *N. gonorrhoeae* is not encapsulated, it rapidly releases small blebs of outer membrane called outer membrane vesicles (OMVs). OMVs contain the Lipid A moiety of LOS and peptidoglycan fragments, both of which are inflammatory and destructive to host tissue (108, 365). OMVs may also act as a decoy for binding of protective host factors (255). Through long-term co-evolution with its human host, the gonococcus has developed many varied mechanisms for the establishment of productive infection, including those described and undoubtedly other mechanisms that remain to be discovered.

Figure 2. Gonococcal virulence factors.

A single gonococcus is illustrated here with the inner and outer membranes depicted by the inner and outer circles, respectively. Virulence factors are shown in correct position with respect to the bacterial membrane and antioxidant defenses are denoted by the dashed box. C' represents complement.

Figure 2. Gonococcal virulence factors.



3.2 Chlamydial Virulence Factors

C. trachomatis is an obligate intracellular parasite and its survival is dependent on uptake and replication within the host cell. For this reason, perhaps the two most important chlamydial virulence factors are the ability to induce phagocytosis by non-professional phagocytes and to inhibit phagolysosomal fusion (Figure 3) (294). The translocated actin-recruiting phosphoprotein (TARP) is injected into the host cell cytoplasm shortly after EB contact through a type three secretion system (T3SS) (51). TARP is phosphorylated in the host cell cytoplasm and then promotes internalization of the EB by an actin-recruiting mechanism (51, 148, 173). Once inside the host cell, chlamydiae are able to inhibit phagolysosomal fusion (95), although the mechanism of inhibition is currently unclear. The fusion inhibitor is thought to be an EB surface antigen (81) and is not present on the RB (33). Additionally, fusion inhibition is specific to the phagosome containing the chlamydiae (80).

Chlamydiae inject several effector proteins into the host cell cytoplasm via a T3SS in order to create a hospitable environment for survival and replication. The chlamydial T3SS is active on the surface of both the EB and the RB and can inject TARP through the host cell plasma membrane, and other effectors, such as the inclusion (Inc) proteins, through the inclusion membrane (22). IncA is an effector protein that localizes to the inclusion membrane (283) and is responsible for inclusion fusion when multiple EBs are taken up into the same cell (111, 326). Natural IncA mutants have reduced virulence (99). Other uncharacterized effector proteins have been shown to translocate from the cytoplasm to the nucleus (132) and are thought to play a role in regulation of NF-κB (22). Several other effector proteins have been identified recently and appear to

be present in pathogenic chlamydiae, but absent in environmental species, suggesting a role in virulence (221). Another interesting potential function of the T3SS is in providing a contact-dependent signal with the inclusion membrane such that the absence of the signal causes the RB to differentiate into the infectious EB form (130).

Two of the most prominent proteins expressed on the surface of the chlamydial EB are highly variable, yet their role in virulence is unclear. The major outer membrane protein (OmpA or MOMP) of *C. trachomatis* is the most prevalent protein on the surface of the EB (41) and exists in as many as 20 different forms (200). While it is not clear why this variability is maintained, it has been hypothesized that it could be due to immune pressure (34, 324), a role in virulence as evidenced by the propensity of certain serovars to be prevalent in specific populations (i.e. homosexual men) (201), differences in the immunogenicity of specific OmpA types, or a link between the *ompA* locus and a gene for an uncharacterized virulence factor that may affect survival or transmission of the organism (39). Like OmpA, the family of polymorphic outer membrane proteins (Pmp) is highly variable and 9 different genes exist in the chromosome that encode these proteins (110). The *C. trachomatis* genome is relatively small and the *pmp* genes account for approximately 14% of the coding content on a nucleotide basis, suggesting an important role in virulence (284). While the exact function of these proteins is currently unclear, several Pmps are expressed on the EB surface and appear to be strongly immunogenic and proinflammatory (330). It is postulated that these proteins have a role in both adherence and modulation of the immune response (352).

In response to certain stressors, such as nutrient deprivation or antibiotic treatment, *Chlamydia spp.* can enter a persistent state *in vitro* (189). In this state, large,

aberrant RBs remain inside the chlamydial inclusion without differentiating into EBs or causing host cell lysis. Following removal of the stressor the chlamydial life cycle resumes and infectious EBs are produced (370). Induction of persistence is known to occur *in vitro* in response to penicillin, interferon-gamma (IFNγ), amino acid starvation, iron deprivation, host cell differentiation state, and coinfection with herpes simplex virus (HSV) (18, 133). The ability of chlamydiae to establish subclinical, persistent infection may be a major contributor to pathogenesis by providing antigen for a continuous pathological immune response and repeat infection, which is a risk factor for development of PID (65). Long-term, sub-clinical chlamydial infection occurs frequently, especially in women, but it is not currently known if this is the result of the organism entering this persistent state *in vivo* or if levels of replication are simply below the limit of detection (19, 370).

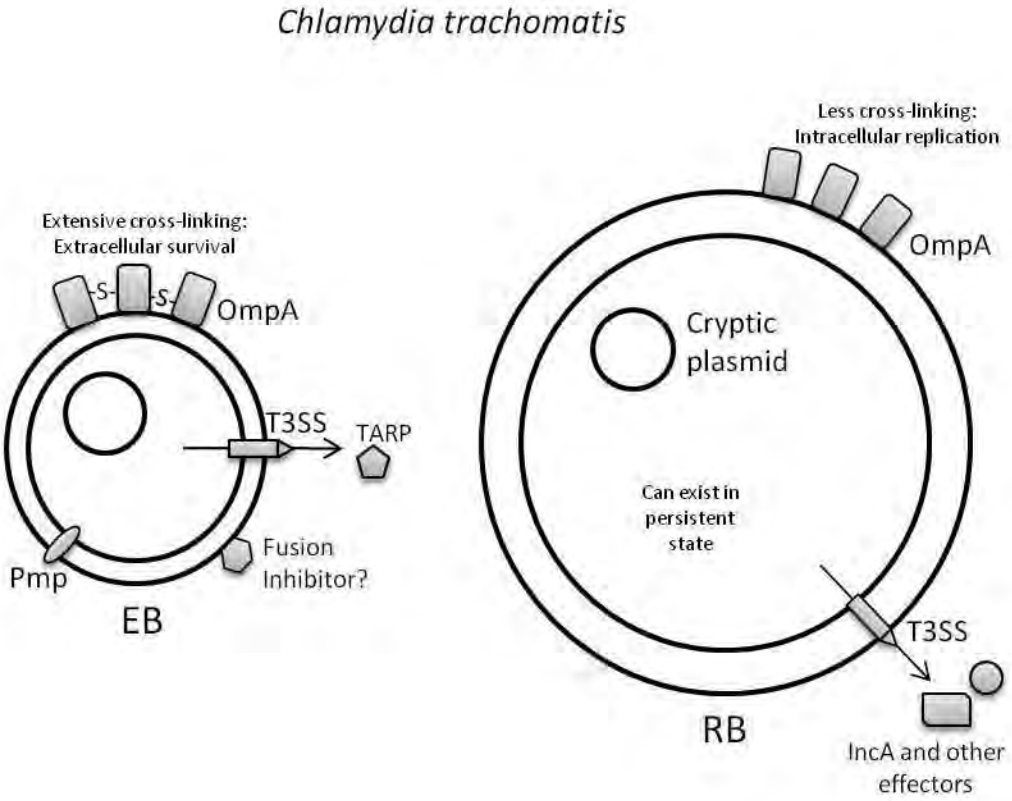
In contrast to the direct tissue damage seen during gonococcal infection (365), most of the pathology that develops in response to chlamydial infection is immune-mediated. For instance, the host response against the chlamydial stress proteins GroEL (Hsp60) and GroES (Hsp10) is associated with disease severity (168, 174, 334, 362). Additionally, most *Chlamydia spp.* contain a cryptic plasmid that is responsible for upper reproductive tract pathology in murine models of infection with *C. muridarum* and *C. trachomatis* LGV strain (229). It is thought that this plasmid encodes for a transcriptional regulator of a chromosomal gene or genes (42). Interestingly, although chlamydiae express lipopolysaccharide (LPS), the lipid A moiety is pentaacylated with longer than usual fatty acid chains (265) and it is unclear whether LPS signaling occurs through toll-like receptor (TLR) -2 or -4 (83). Purified chlamydial LPS results in only

weak macrophage activation (123) and thus it is unclear whether LPS is a major inducer of genital pathology. However, in high concentrations, EBs themselves are toxic and if a host cell ingests too many EBs, it will die even without replication of the organism (162).

Figure 3. Chlamydial virulence factors.

The two forms of chlamydiae, the elementary body (EB) and reticulate body (RB), are shown on the left and right, respectively. The inner and outer circles represent the inner and outer membrane, respectively. TARP is injected through the host cell plasma membrane into the host cell cytoplasm by the EB. IncA and other effectors injected into the host cell by the T3SS are injected through the inclusion membrane and may localize to the inclusion membrane, the host cell cytoplasm, or the nucleus.

Figure 3. Chlamydial virulence factors.



3.3 Host Response to Gonococcal Urogenital Infection

The innate immune response to gonococcal urogenital infection has been characterized based on human studies, the female mouse model of infection, and cell culture systems (Figure 4). Symptomatic gonococcal infection in humans is characterized by a mucopurulent discharge consisting of polymorphonuclear leukocytes (PMNs) that often contain intracellular gonococci, extracellular gonococci, and desquamated epithelial cells (344). This discharge is absent during asymptomatic infection. A proinflammatory cytokine response occurs *in vitro* upon exposure of several different epithelial cell types to *N. gonorrhoeae*, including vaginal, endocervical, ectocervical (91, 222), urethral (116), endometrial (50), and fallopian (207) cells. In a fallopian tube organ culture system, gonococcal infection results in the production of proinflammatory mediators interleukin (IL) -1α, IL-1β, and tumor necrosis factor-alpha (TNFα) (184). Professional phagocytic cells, including primary macrophages and monocytes, also produce proinflammatory cytokines in response to *N. gonorrhoeae* (185, 244, 278), which may serve to further amplify the epithelial response. In support of these *in vitro* data, experimental infection of male volunteers with *N. gonorrhoeae* results in the localized production of inflammatory cytokines (268, 269), however, localized production of inflammatory cytokines in naturally infected women was not detected (121, 122). During gonococcal infection of female BALB/c mice, several inflammatory cytokines and chemokines including TNFα, IL-6, and macrophage inflammatory protein 2 (MIP-2) and keratinocyte-derived cytokine (KC), which are analogs of the human chemoattractant IL-8, were elevated in vaginal washes from infected mice compared to uninfected controls at day 5 post-infection (236). It is not known why some gonococcal

infections are symptomatic and others are asymptomatic. However, differences in the susceptibility of different mouse strains to gonococcal infection or the production of an inflammatory response during infection suggest that host genetic factors may play a role in determining susceptibility and immune response (236).

Despite a seemingly robust innate response to gonococcal infection, the adaptive and memory responses to infection are relatively weak. Levels of *N. gonorrhoeae*-specific antibodies in the genital secretions and serum of naturally infected individuals are low or undetectable and appear to be transient, with greater levels during acute infection than during convalescence (52, 141, 157, 194-196, 232, 335, 374). A similar transient and insignificant antibody response was observed following gonococcal challenge in female mice and upon a second challenge with the same strain, there was no protection against reinfection nor was there a boost in antibody titer (317). Several studies have found no protective immunity against reinfection with *N. gonorrhoeae* even with the same serotype (31, 93, 131, 287), suggesting a lack of immunological memory, although there is evidence that repeated infection may induce some partially protective, strain-specific immunity (36, 260, 296). It seems unlikely that the reason for the weak humoral response to gonococcal infection is the lack of inductive sites in the genital tract (121). Immunization of female mice with gonococcal OMVs at a location with organized lymphoid tissue (intranasally) results in a decreased duration of gonococcal colonization compared to non-immunized controls (257), suggesting that a protective immune response can be generated locally in the genital tract. However, during natural infection, even when gonococci are present at sites with local organized lymphatic tissue, such as the rectum, there are still only low levels of gonococcal antibodies present (121).

Furthermore, intravaginal immunization with certain antigens has been demonstrated to evoke a robust vaginal and cervical antibody response (172, 317).

Due to the unimpressive nature of the humoral response to gonococcal infection and the lack of a protective, immunological memory response, it has been postulated that *N. gonorrhoeae* may actively suppress the adaptive immune response to infection, despite the strong adjuvant activity of neisserial porin (187). In patients with gonorrhea, a transient decrease in the number of circulating CD4+ T cells is observed that resolves after clearance of infection (6). The engagement of *N. gonorrhoeae* Opa proteins with CEACAM1 on the surface of CD4+ T cells inhibits T cell proliferation and activation (26) and suppresses the adaptive immune response by preventing T cell receptor activation (176). Additionally, in the female mouse model, Imarai and colleagues demonstrated the generation of regulatory T cells at the site of infection, including both TGFβ+ CD4+ T cells and CD25+ Foxp3+ T cells (140). Taken together, these reports suggest that *N. gonorrhoeae* has evolved several mechanisms for suppressing the host immune response.

Recent advances have been made in understanding the immunological pathways stimulated during gonococcal infection and in defining the pathways that are protective. Gonococcal LOS is an agonist of TLR4 (262) and our lab recently showed that BALB/c-*LPS*^{d} mice, which are deficient in TLR4-signaling, are less able to control gonococcal infection as evidenced by increased recovery of gonococci from the genital tract. These results suggest TLR4-mediated responses are protective for gonococcal genital tract infection in mice (237). Additional studies using the mouse model of gonococcal genital infection revealed that gonococci induce a Th17-type response, which is protective and is

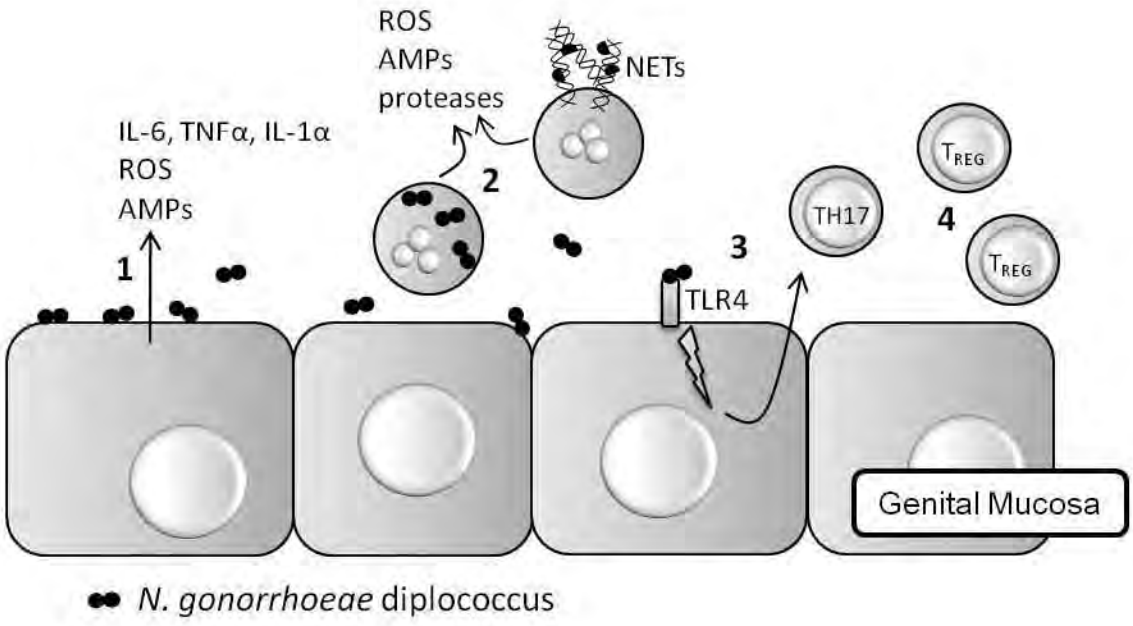
characterized by the release of IL-17 and the recruitment of PMNs (88). In support of these findings, Gagliardi and colleagues showed that patients with gonococcal urethritis and pharyngitis had increased levels of serum IL-17A and IL-23, which are important for the differentiation and proliferation of Th17 cells (97). Interestingly, it was recently shown that signaling of *N. gonorrhoeae* through TLR4 is linked to the induction of protective Th17-type responses (88), providing a potential mechanism for TLR4-mediated protection. In addition to PMN recruitment, Th17-type responses are also characterized by the production of antimicrobial peptides (AMPs) (96, 372), several of which have activity against *N. gonorrhoeae* in *in vitro* assays (263, 308, 351, 373).

Several studies suggest that hormones may play a role in modulating the host response to gonococcal infection. Initial studies from the late 1960s and 1970s demonstrated changes in culture rates of *N. gonorrhoeae* from women depending on stage of the menstrual cycle or oral-contraceptive use (143, 153). Similar fluctuations were later observed in the female mouse model of genital infection (311) and were shown to be dependent on the presence of the ovaries, and thus reproductive hormones (56). The female genital tract contains many different potentially bactericidal factors that are only just beginning to be characterized with respect to effect on sexually transmitted pathogens (55). Some of these factors, including AMPs (310), and innate immune signaling molecules such as TLR4 (129) are hormonally regulated and may play an important role in the defense against gonococcal infection.

Figure 4. *Host response to urogenital gonococcal infection.*

(1) Upon infection with *N. gonorrhoeae*, genital epithelial cells produce several proinflammatory cytokines including IL-6, TNFα, and IL-1α, as well as reactive oxygen species (ROS) and antimicrobial peptides (AMPs). (2) PMNs migrate to the site of infection and produce many antibacterial effectors including ROS, AMPs, proteases, and neutrophil extracellular traps (NETs). However, gonococci are able to survive and replicate within PMNs. (3) Gonococcal signaling through TLR4 appears to play a protective role during gonococcal infection and may be important for induction of a Th17 response. (4) Regulatory T cells are detected at the site of infection in a murine model of gonococcal infection.

Figure 4. Host response to urogenital gonococcal infection.



3.4 Host Response to Chlamydial Urogenital Infection

A complete discussion of the host response to chlamydial infection requires consideration from two different perspectives. In the case of genital chlamydia, the host must balance the immune response very carefully if it is to clear the infection without damage to the genital tract, as most of the pathology associated with chlamydial infection is immune-mediated (65). It is, therefore, of particular importance that the distinction between protective versus pathologic immune responses be made.

Several facets of the host immune response are consistently detected during chlamydial infection in humans, as summarized by Geisler (98). High levels of serum and genital mucosal antibodies specific to certain *C. trachomatis* proteins and *C. trachomatis* EBs are usually detected (4, 102, 202, 243). Levels of several proinflammatory cytokines can be found in increased concentrations in genital secretions (4, 243, 349). PMN counts in the male urethra (100) and female cervix (103) are not consistently elevated, however, in women there is a consistent increase in the number of T cells found locally in the cervix, but not systemically (203). Finally, mononuclear cells collected from the blood or the genital mucosa of *C. trachomatis*-infected patients tend to display lymphoproliferative responses when exposed to chlamydial antigens (4, 203). As discussed, one of the major concerns during chlamydial infection is the development of PID in women and damage to the host tissue that can cause permanent scarring and fibrosis. Unfortunately, it has been difficult to determine the precise mechanism of chlamydiae-induced tissue damage due to the prominence of both acute and chronic inflammatory cells in biopsies from women with PID (170).

Based on mouse and guinea pig models of genital chlamydiae infections in combination with *in vitro* cell culture studies, many elements of the host response to chlamydial infection have been well characterized (Figure 5). The host response to primary infection usually occurs within 1-2 days and is characterized by mucosal infiltration by some monocytes, but predominantly PMNs, which can kill accessible EBs in the extracellular space (63, 164, 210, 316). This response is initiated by the release of IL-1β from *C. trachomatis*-infected epithelial cells, which serves to amplify the proinflammatory cytokine response and elicits the production of TNFα, IL-8, growth-relaetd oncogene-alpha (GROα), granulocyte-macrophage colony stimulating factor (GM-CSF), and IL-6 (20, 63, 66, 139, 279). Interestingly, attachment and uptake of the EB by the host cell is not sufficient to initiate this response and intracellular chlamydial replication is required for IL-1β release (279). The production of proinflammatory cytokines by genital epithelial cells, and to a lesser extent by resident tissue macrophages, results in the rapid recruitment of immune cells, including PMNs, natural killer (NK) cells, and monocytes, to the site of infection (65). Epithelial cells and PMNs express a number of matrix metalloproteinases (MMPs) and elastases which contribute to tissue damage at the site of infection (65). Later, T cells begin to accumulate at the site of infection and many studies have emphasized the importance of the development of a T cell response for controlling infection (209, 267, 271). During early chlamydial infection in mice, Th17 and Th1 CD4+ T cells are present to a similar extent potentially due to the presence of both IL-1β, which drives the differentiation of Th17 cells, and IFNγ, which drives the differentiation of Th1 cells (302). NK cells present in the genital tract during infection produce large amounts of IFNγ, which eventually skews the CD4+ T cell

differentiation toward an exclusively Th1-type response (302). In an effort to resolve infection, a mixture of CD4+ and CD8+ T cells, B cells, and antibody secreting plasma cells infiltrate the infected tissue (65). Plasma cells release antibodies that can inactivate extracellular EBs (65) and T cell-produced IFNγ can inhibit chlamydial cellular replication (252). Several studies have demonstrated the dependence of infection resolution on IFNγ (40, 60, 209, 210, 251). Inflammation subsides once the infection has been resolved, but chronic scarring may already have occurred if infection was able to ascend to the upper reproductive tract.

Chlamydial infection that ascends to the upper reproductive tract in women is of particular concern due to the risk for complications. In mice, tissue damage frequently occurs as a result of primary infection, suggesting that the inflammatory process generated during a single chlamydial infection may be enough to induce permanent damage (316). In guinea pigs, however, primary infection results in long-term damage in only a minority of animals (274). Data on the frequency of clinical complications following primary infection in women is limited, but it is thought that the majority of primary infections do not result in complications (234, 340), suggesting that the guinea pig model of chlamydial infection may be more similar to human disease progression in terms of the development of upper reproductive tract disease. Chronic (206) and repeat (37) infections are frequent in women and are thought to lead to a greater incidence of tissue scarring. This hypothesis is supported in both primate (342) and guinea pig (275) models of chlamydial infection, in which chlamydiae-specific CD4+ and CD8+ T cells infiltrate more rapidly and in greater numbers during repeat oviduct infection causing enhanced disease that requires very few bacteria. Current consensus in the field is that

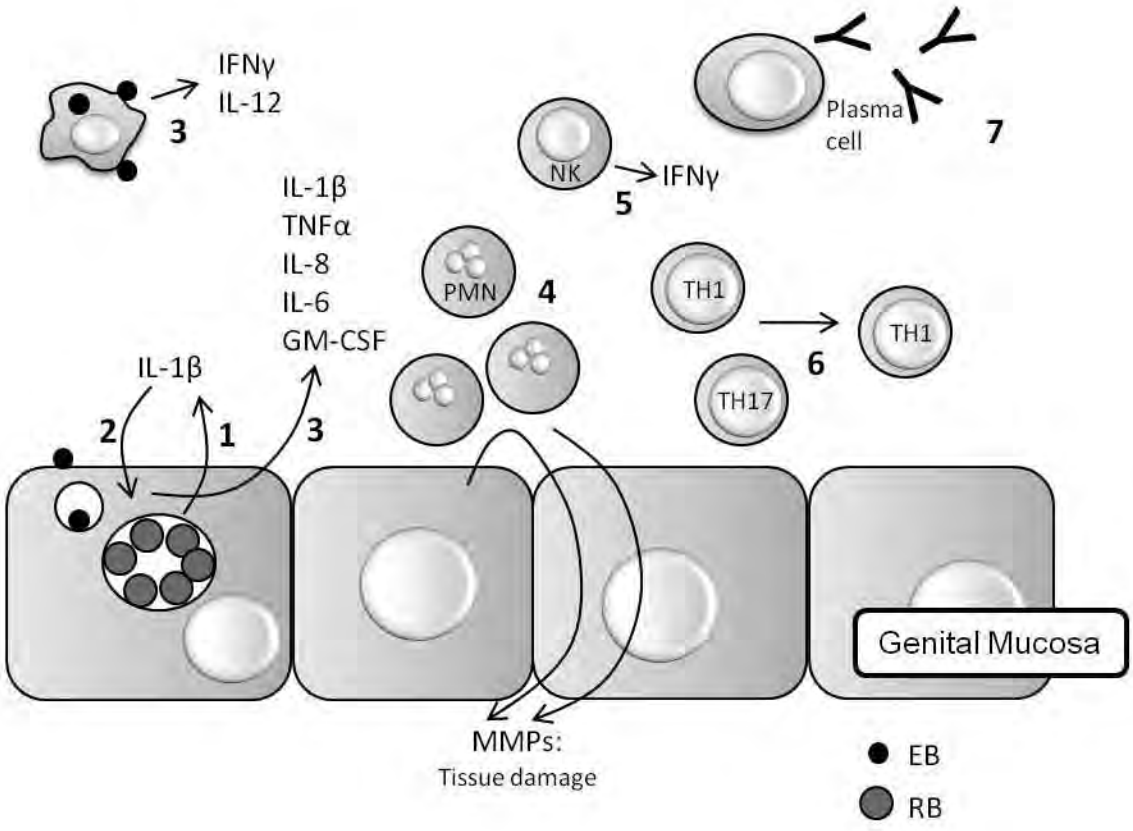
chlamydiae must ascend to the upper genital tract and cause infection of the oviduct epithelium in order to cause long-term sequelae (65) and that the Th1 skew of the initial adaptive and memory responses is protective against sequelae (2).

Chlamydiae are recognized by both TLR2 and TLR4, although interaction with TLR4 *in vitro* appears to be minimal (230). Interaction of the organism with TLR2 is dependent on intracellular replication and TLR2 co-localizes with the chlamydial inclusion, suggesting signaling from within the cell (230). Initial recognition by the host *in vivo* also appears to be dominated by TLR2 (154) and chlamydial signaling through TLR2 is linked to the development of immunopathology in mice (67, 229). Interestingly, a plasmid-cured strain of *C. muridarum* which cannot signal through TLR2 is competent for infection in mice and provides immunological resistance upon repeat infection; however, no pathology develops in the upper genital tract upon initial infection with the plasmid-cured strain or repeat infection with a wild type strain (229). This finding is of particular importance because it demonstrates the ability of the host response to effectively clear chlamydiae from the upper genital tract without the development of pathology and to prevent pathology from developing during a repeat infection, providing hope for the development of an effective vaccine (65).

Figure 5. Host response to urogenital chlamydial infection.

(1) IL-1β is released by genital epithelial cells following chlamydial replication within the host cell. (2) IL-1β acts on host epithelial cells to amplify the proinflammatory response. (3) Several proinflammatory cytokines and chemokines are released from host epithelial cells and resident macrophages. (4) PMNs migrate to the site of infection and release proteases and matrix metalloproteinases (MMPs), which can cause tissue damage. Epithelial cells also produce damaging MMPs. (5) Natural killer (NK) cells migrate to the site of infection and produce large amounts of IFNγ. (6) During initial infection Th1 and Th17 CD4+ T cells are present in the genital tract in approximately equal numbers, but as infection proceeds and more IFNγ is produced the response becomes skewed toward an exclusively Th1 response. (7) Plasma cells produce chlamydiae-specific antibodies, which are detectable in the genital tract and systemically.

Figure 5. Host response to urogenital chlamydial infection.



4.0 In vivo *Models*

4.1 Experimental Models of Gonorrhea

The development of *in vivo* models for studying the pathogenesis of the strict human-specific pathogen *N. gonorrhoeae* has been difficult. One of the most useful models of gonorrhea utilizes experimental infection of male volunteers (53). In this model, male subjects are inoculated intraurethrally with gonococci. Symptoms of gonorrhea usually develop within 2-4 days and volunteers are treated immediately upon development of symptoms (54). This model has been used with much success for the study of many virulence factors including opacity proteins (145, 296), pilin antigenic variation (114, 306), LOS (297, 298), sialylation (299), iron acquisition (5, 58), and IgA1 protease (149). The human model has also been useful for characterizing the inflammatory response to infection (268), susceptibility to reinfection (296), and testing of vaccine candidates (25, 54). Despite the success of this model, the male urethra is not biologically representative of the female genital tract and women cannot be experimentally infected due to the risk for complications. Additionally, mechanistic studies cannot be performed on human subjects. Therefore, animal modeling of gonococcal infection remains the only option for the study of gonococcal infection in the female genital tract and for performing mechanistic studies in which the host can be manipulated.

The first animal models of gonococcal infection were developed in the 1930s and 1940s and included intraperitoneal inoculation of mice and intraocular inoculation of rabbits (7, 198). Renewed interest in the animal modeling of gonococcal infection in the

1970s and 1980s resulted in several models of both systemic and localized infection. Models of infection included direct synovial inoculation in rats (92) and rabbits (105) to mimic gonococcal arthritis and intraperitoneal inoculation in rodents to mimic DGI (226). Studies using inoculation of subcutaneous chambers implanted in guinea pigs and rats provided information about the interaction of *N. gonorrhoeae* with PMNs and antigenic variation *in vivo* (8, 9, 12, 48). Male chimpanzees that were inoculated with *N. gonorrhoeae* developed urethritis similar to that seen in men (180) and sexual transmission from male to female chimpanzees was observed (32). Early vaccine studies were also conducted in chimpanzees (10, 11). Importantly, gonococcal infection of chimpanzees was the first animal model that allowed for the study of genital tract infection. The success of gonococcal infection in chimpanzees served to underscore the importance of several host-restricted factors necessary for gonococcal colonization, which are limited to primates. Unfortunately, due to financial constraints and limited availability, chimpanzees are not a reasonable model for gonococcal research (7).

Several laboratories attempted to establish genital colonization with *N. gonorrhoeae* in female mice and it was observed that mice were only transiently susceptible to gonococcal infection during certain stages of the estrous cycle, when levels of estrogen were high and progesterone were low (Figure 6) (62, 152, 169). In 1990, David Taylor-Robinson described long-term gonococcal colonization following treatment of germ-free mice with 17β-estradiol (331). Our laboratory further refined this technique and established a female mouse model of gonococcal infection in which long-term colonization is established following treatment of mice with 17β-estradiol and antibiotics to control overgrowth of commensal flora (144). Initial studies were conducted using

subcutaneous implantation of a slow-release estradiol pellet, but the model has since been refined with the use of subcutaneous injections of water-soluble estradiol, which allows a return to physiological concentrations of estradiol in the serum within 24 hours of treatment, thus subjecting the gonococci to artificially high levels of estradiol for a shorter period of time (144, 317). Several other groups have since adapted this model for their own purposes, using slightly different vehicles for estradiol administration (27, 140, 215). In mice treated with water-soluble estradiol, gonococcal infection lasts an average of 10 days with an average range of 10^3 to 10^5 colony forming units (CFUs) recovered by daily vaginal swab and a PMN influx on days 5-8 post-infection in approximately 50% of mice (317). Gonococci are detected deep within the vaginal and cervical tissue (317) and ascend to the upper genital tract in approximately 18-20% of mice (144). Infected mice have an inflammatory response that is characterized by PMN influx and the induction of TNFα, IL-6, MIP-2, and KC (144, 236). Similar to human infection, mice develop an unremarkable humoral response to infection that leaves the mice susceptible to repeat infection with the same strain (144, 236, 317).

Numerous host restrictions limit the capacity of female mice to fully mimic gonococcal infection in humans. For example, the pilin and opacity (Opa) protein receptors CD46 and carcinoembryonic antigen-related cell adhesion molecules (CEACAMs), respectively, are host-restricted (56, 156, 213, 345) as are interactions with soluble complement regulatory proteins (225), *N. gonorrhoeae* cannot use non-human lactoferrin or transferrin as sources of iron (57, 175) and the gonococcal IgA1 protease cannot cleave mouse IgA (166). Despite these host restrictions, studying gonococcal pathogenesis in the murine model has yielded considerable insight into the host response

to infection (88, 140, 236, 317) and the role of certain gonococcal virulence factors in evasion of host defenses (146, 315, 350, 367, 368). The use of female mice to study gonorrhea has also revealed interesting hormonal influences on antigenic variation of Opa proteins *in vivo* (56, 144, 311), the effect of certain antibiotic resistance mutations on microbial fitness (351), and new information on the interaction between gonococci and H_2O_2-producing commensal flora *in vivo* (214). The availability of transgenic mice in several of these host-restricted factors, including transferrin (375) and CEACAMs (215), will allow for improved study of gonococcal pathogenesis *in vivo*. For example, human CEACAM transgenic mice were recently used by Muenzner and colleagues to demonstrate a novel role for Opa-CEACAM interaction in inhibiting epithelial sloughing during gonococcal infection (215). Refinements such as these allow for continued improvement of the model and more detailed study of the complex interactions between *N. gonorrhoeae* and the female host in the context of an intact immune system and vaginal flora.

4.2 Experimental Models of Chlamydia

Several models of chlamydial infection have been developed and have been instrumental in understanding the pathogenesis and immunobiology of chlamydia. The most widely used models include non-human primates, pigs, guinea pigs, and mice.

Many different non-human primate species have been used to study chlamydial infection including marmosets, grivets, and pig-tailed macaques (277). Pig-tailed macaques have been the most useful non-human primate model for several reasons including size, quiet temperament, well-characterized menstrual cycle, a genital tract anatomy and physiology that is similar to humans, and natural susceptibility to human

strains of *C. trachomatis* (364). Macaques develop a chronic chlamydial infection with intermittent shedding. The initial response to infection is PMN based (151), then mononuclear (245), but eventually a Th1-type response develops (341) that consists of primarily CD8+ T cells (342).

Genital chlamydial infection has been established in pigs using *C. trachomatis* serovar E (343). The local genital infection results in symptoms including development of erythamatous vulva and fever, suggesting a systemic response to infection. Shedding of the organism can be detected for up to 21 days and it is thought that the pig model may have value for studying hormonal influences on infection (343).

Chlamydial infection of guinea pigs is established using *C. caviae*, or the guinea pig inclusion conjunctivitis (GPIC) strain. Infection of guinea pigs with *C. caviae* results in a self-limited infection of 3-4 weeks in duration that can be established in both males and females, allowing for the study of sexual transmission (220, 272). Ascendant infection occurs in approximately 80% of guinea pigs (274). Guinea pigs have a relatively long estrus phase of the estrous cycle, which allows for studies of hormonal influence on infection and vertical transmission from mothers to pups has been demonstrated (212). While the guinea pig model of chlamydial infection has been very useful, there are some difficulties due to the limited availability of reagents for studying the immune response.

A number of mouse models of chlamydial infection exist. In 1981, Barron *et. al.* described inoculation of female mice with what was then known as the *C. trachomatis* agent of mouse pneumonitis (MoPn) (15) and has since been reclassified as *C. muridarum* (85). It was determined that susceptibility of mice to chlamydial infection is

highly dependent on stage of the estrous cycle, and in contrast to susceptibility of mice to infection with *N. gonorrhoeae*, mice are most susceptible to chlamydial infection when progesterone levels are high during the diestrus stage (Figure 6) (15, 21, 270). To overcome the need to determine stage of the estrous cycle prior to inoculation, two methods of *C. muridarum* inoculation were developed. Researchers either inoculate with *C. muridarum* on 2-3 consecutive days in order to ensure that mice are inoculated on a susceptible day of the estrous cycle (15, 270) or treat with progesterone prior to inoculation in order to synchronize mice in a diestrus-like state (21). Models of infection of female mice with human *C. trachomatis* serovars have also been developed and characterized (270). In 1982, Tuffrey and Taylor-Robinson described establishment of infection with human strains of *C. trachomatis* following intra-uterine inoculation (338). In general, however, mouse models that use human strains of *C. trachomatis* require higher inocula, have a lower peak bacterial load, and demonstrate relatively quick eradication of infection compared to infection with *C. muridarum* (252). Moreover, pathology is limited to the lower genital tract and there is a more pronounced delay of clearance in IFNγ or IFNγ-receptor knock-out mice (142, 150), likely due to a discordance in the IFNγ-response of the host with the IFNγ-defenses of the organism (190, 286). Upper genital tract pathology has only been demonstrated following direct inoculation of large numbers of *C. trachomatis* into the uterine horns or ovarian bursa (336, 337). The course of *C. muridarum* infection in female mice seems to be more similar to human genital chlamydial disease than infection of mice with human serovars of *C. trachomatis*. *C. muridarum* naturally ascends from the lower to the upper genital tract of female mice (15) and causes an inflammatory response and post-infection

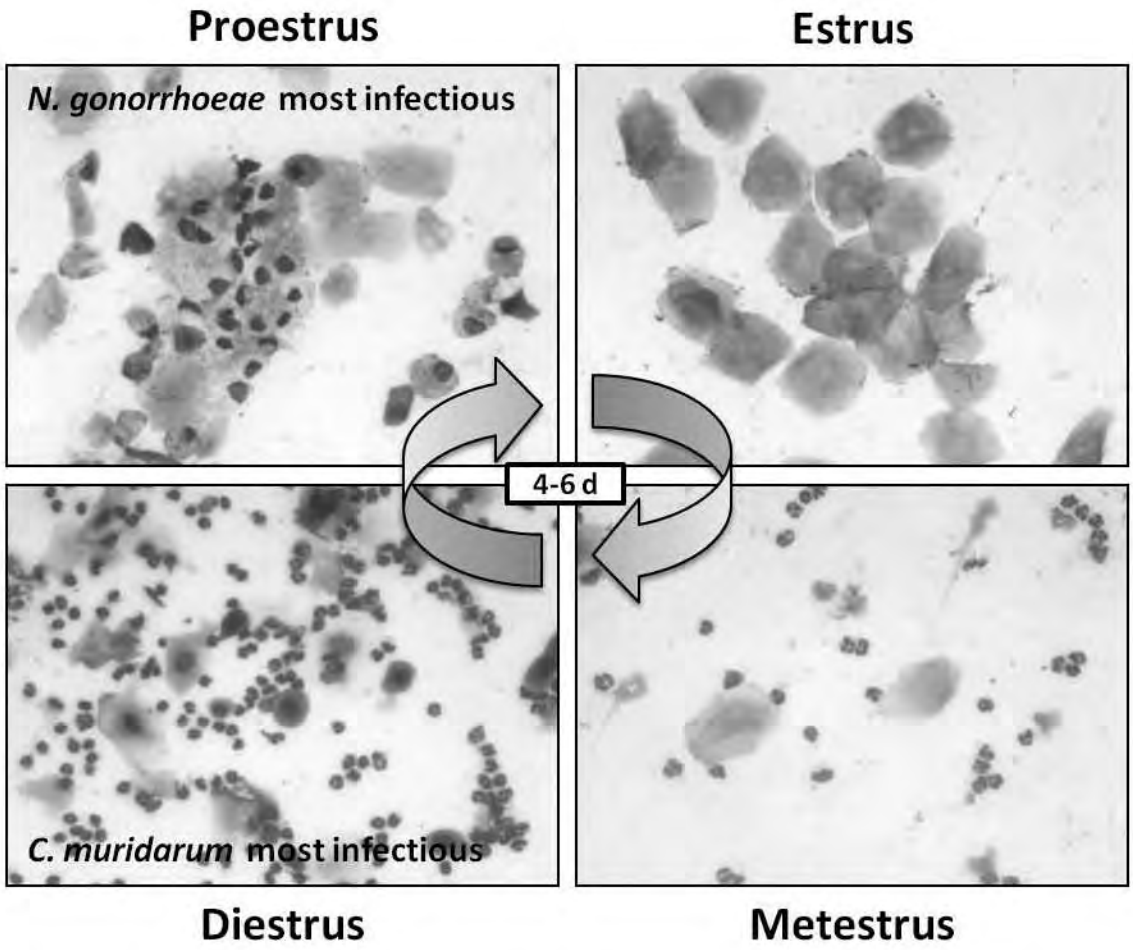
sequelae, such as tubal occlusion, hydrosalpinx, and infertility, similar to those seen in women (360). As mentioned, most researchers who use the *C. muridarum* model treat the mice with progesterone, which increases the rate of infection and the number of inclusions recovered, but does not alter the duration of infection (15, 59). Studies done in different mouse backgrounds have demonstrated that severity of infection varies depending on mouse strain as measured by bacterial recovery and development of pathology (63). Examination of differences between strains has been informative in determining host factors important for controlling infection (64). Interestingly, infection with *C. muridarum* can also be established in male mice (239), which suggests the potential for developing a model of transmission.

In summary, the availability of several good animal models of chlamydial infection has allowed for an exceptional understanding of chlamydial pathogenesis and immunobiology. By combining the data collected from animal models with clinical studies in which human samples are collected researchers can try to determine which animal model best suits the study of different aspects of chlamydial pathogenesis and can thus use the best model available to answer specific questions.

Figure 6. Female mice are differentially susceptible to N. gonorrhoeae *and* C. muridarum *during different stages of the estrous cycle.*

Stained vaginal smears are shown from mice in the four different stages of the estrous cycle, which lasts approximately 4-6 days. Mice are most susceptible to gonococcal infection when levels of estradiol are high during proestrus, which is characterized by the predominance of nucleated epithelial cells and some squamous cells. In contrast, mice are most susceptible to chlamydial infection when levels of progesterone are high during diestrus, which is characterized by nucleated epithelial cells and large numbers of PMNs.

Figure 6. Female mice are differentially susceptible to N. gonorrhoeae *and* C. muridarum *during different stages of the estrous cycle.*



4.3 Modeling Polymicrobial Interactions in the Genital Tract

Experimental models of coinfection have become increasingly common as researchers realize the necessity of accurately representing the complex, polymicrobial interactions that take place *in vivo*. Due to the presence of normal flora and the propensity for coinfection with multiple sexually transmitted pathogens, it is especially important to model polymicrobial interactions when studying sexually transmitted infections in the female genital tract. Two such models of genital co-colonization have been described. Based on models of single pathogen infection, it was hypothesized that cell-mediated immunity to *Candida albicans* would be boosted by chlamydial coinfection, however, the two genital infections proceeded independently and neither disease was altered by the other in a murine model of coinfection (163). Similarly, despite the ability of hydrogen peroxide-producing *Lactobacillus crispatus* to inhibit growth of *N. gonorrhoeae in vitro*, there was no effect of lactobacilli on gonococcal colonization or duration of recovery during co-colonization in the genital tract of female mice, even when gonococcal mutants in antioxidant defenses were tested (214). These findings suggest that many of the complex interactions that occur in the genital tract are not modeled well *in vitro* and cannot always be deduced from single pathogen infection.

As discussed, pathogenesis and host response differ greatly during infection with either *N. gonorrhoeae* or *C. trachomatis*, most notably with respect to cellular localization of the bacteria and polarization of the immune response toward a strong Th1 phenotype during chlamydial infection (65) and a Th17 phenotype during gonococcal infection (88, 97). Thus, it is reasonable to expect that gonococcal and chlamydial coinfection may differ in many aspects from infection with either *N. gonorrhoeae* or *C.*

trachomatis alone, yet little is currently known about the ways in which coinfection is similar to or different from infection with either pathogen alone.

5.0 Gonococcal and Chlamydial Coinfection

5.1 Current Knowledge about the Pathogenesis of Coinfection

Excellent mouse models of both gonococcal and chlamydial infection already exist and have been used extensively in order to better understand the pathogenesis and host response that occurs during gonorrhea and chlamydia (15, 144, 270, 317). The development of a coinfection model that is based on combining these single pathogen models into a model of gonococcal and chlamydial coinfection in the female genital tract would be a major step toward understanding the pathogenesis of gonococcal and chlamydial coinfection.

All studies to date of gonococcal and chlamydial coinfection are epidemiologic in nature and most studies have sought to determine the rates of coinfection in different populations, as described above. However, some aspects of pathogenesis and the immune response have been studied. In some instances, strains of *C. trachomatis* or *N. gonorrhoeae* isolated from coinfected patients and patients infected with either pathogen alone were serotyped in an effort to correlate certain strains with increased incidence of coinfection. In two such studies of heterosexual men in Italy and Greece, increased rates of gonococcal coinfection were associated with *C. trachomatis* serovars D or J, respectively (74, 240). In both studies, the overall rate of *N. gonorrhoeae* coinfection was approximately 30%; however, despite a similar distribution of chlamydial serotypes in both populations, coinfection was associated with two different serotypes and thus no

clear correlation between *C. trachomatis* serotype and gonococcal coinfection can be made. Conversely, in a study performed in Stockholm in which a 20% rate of chlamydial coinfection was observed in heterosexual men infected with *N. gonorrhoeae*, gonococcal isolates were characterized based on porin type and PIA strains were more frequently associated with chlamydial coinfection in this population (288). A similar study conducted in Scotland over a four year period yielded a slightly higher rate of coinfection of approximately 35% and was unable to detect an association of gonococcal porin type with chlamydial coinfection, although only women were surveyed making the two studies difficult to compare directly (192). At present, there is no clear correlation of any gonococcal or chlamydial factors with coinfection based on limited characterization of isolates from coinfected individuals.

Another major area of interest is whether coinfection alters the ability of *N. gonorrhoeae* or *C. trachomatis* to be transmitted from a coinfected individual to an uninfected sexual partner. Three studies have attempted to answer this question and no clear difference in transmission of either organism in the case of coinfection was observed (179, 181, 188). However, a major difficulty in performing such studies is appropriate identification of the index case. In several cases, partners were infected with a pathogen that the suspected index case was not, begging the question, "Who had it first?" Therefore, in a letter published in the journal *Genitourinary Medicine* in 1995, Matondo and colleagues expressed the need for further study before conclusions about transmission in the context of coinfection can be made (188).

C. trachomatis is known to cause long-term, chronic infection, especially in women where untreated, asymptomatic infection is thought to last an average of 1.5 years

(86). *In vitro* studies suggest that chlamydiae can exist in a persistent form within the host cell following periods of stress, such as antibiotic treatment or nutrient deprivation (189). During long-term, chronic infection it is not known whether infection is merely subclinical, or if the organism is actually able to exist in this persistent form within the host (370). Two epidemiologic studies suggest that coinfection with *N. gonorrhoeae* may serve to reactivate latent chlamydial infection in women. Batteiger *et. al.* described a significant increase in the rate of recurrent chlamydial infection, defined by isolation of the same chlamydial serovar during primary and recurrent infection, in women coinfected with *N. gonorrhoeae* (17). Similarly, Lin and colleagues observed an 80% rate of chlamydial coinfection in women who were exposed to male sex partners infected with *N. gonorrhoeae* alone, suggesting that *N. gonorrhoeae* may allow for latent, sub-clinical chlamydia to flourish (179).

Many symptoms of both gonococcal and chlamydial infection, such as dysuria and discharge, are very similar, although rates of asymptomatic infection with both organisms are high. In an unselected adolescent population, reports of symptoms were significantly greater in coinfected individuals (16% reported symptoms) than in individuals infected with either pathogen alone (8% of *N. gonorrhoeae* infected and 5% of *C. trachomatis* infected reported symptoms) (227). Similarly, in a study of women infected with *C. trachomatis* attending genitourinary medicine clinics in the United Kingdom, women who were coinfected with *N. gonorrhoeae* were approximately 20% more likely to report symptoms than women infected with *C. trachomatis* alone (285). Whether or not this increased symptomology may indicate a greater risk for development of complicated disease is unknown; however, there is some indication that coinfected

individuals have an increased risk for the development of tubal factor infertility. In Gambia, sera tested for the presence of antibodies specific for *C. trachomatis* and *N. gonorrhoeae* were more frequently positive in infertile women than in matched, pregnant controls and strikingly, 54% of infertile patients were positive for antibodies against both pathogens (183). A larger study performed by the World Health Organization Task Force on the Prevention and Management of Infertility and consisting of centers in Thailand, Slovenia, and Hungary found that the risk for infertility caused by bilateral tubal occlusion was highest in women who had serological evidence of infection with both *C. trachomatis* and *N. gonorrhoeae* (357). Although sera cannot demonstrate the existence of the two infections simultaneously, these data do raise the question of whether gonococcal and chlamydial coinfection results in increased risk for upper reproductive tract damage and ultimately infertility.

5.2 Unanswered Questions about Coinfection

Epidemiological data on gonococcal and chlamydial coinfection are limited and no *in vitro* or *in vivo* model systems of coinfection have been described. Thus, there are many unanswered questions about coinfection relating to disease susceptibility and transmission, disease severity and the development of complications, and bacterial and host factors that may favor coinfection.

Three potential mechanisms of establishing gonococcal and chlamydial coinfection exist. An individual may be infected first with *C. trachomatis* followed by *N. gonorrhoeae*, infected first with *N. gonorrhoeae* followed by *C. trachomatis*, or infected simultaneously with both organisms by a coinfected partner. Based on the ability of *C. trachomatis* to cause long-term, asymptomatic infection (86) and the high frequency of

coinfection (366), one might postulate that the first and third possibilities described are most common, although this is not known. Additionally, it is not known if infection with one pathogen may increase the susceptibility of the host to the other, potentially by altering the local host immune response or remodeling the genital tract in a way that exposes more colonization receptors or susceptible cells. Despite attempts at determining the effects of coinfection on transmission (179, 181, 188), there is still no clear evidence that coinfection can alter the ability of a coinfected host to transmit infection to an uninfected partner. If coinfection does alter pathogen transmission, there may be gender differences as generally, transmission of STIs occurs more efficiently during vaginal intercourse from the male to the female (82).

It is important to understand the effect of gonococcal and chlamydial coinfection on disease severity and the development of complicated upper reproductive tract disease. The mechanism by which *N. gonorrhoeae* and *C. trachomatis* cause upper reproductive tract tissue damage in women is not completely understood, although some theories have been put forth and tubal scarring appears to require ascension of the organisms. *N. gonorrhoeae* damages tissue directly and *C. trachomatis* damage is immune-mediated, therefore coinfection may change the way one organism or the other behaves in the upper reproductive tract and this could impact the design of immunological therapies for the treatment of PID (248). Limited data exist to suggest that infection with *N. gonorrhoeae* and *C. trachomatis* either simultaneously or at different times increases the likelihood of complicated infection and eventual infertility (183, 357). Studies on the presence of disease symptoms demonstrated that coinfection is more frequently symptomatic than

infection with either pathogen alone (179, 285), which may also suggest a greater level of tissue damage, although these theories need to be tested experimentally.

Isolates of *N. gonorrhoeae* and *C. trachomatis* from coinfected individuals have not been well-characterized, but some attempts were made to correlate bacterial factors such as chlamydial OmpA and neisserial porin with increased rates of coinfection as discussed (74, 192, 240, 288). Many more bacterial factors remain to be tested, however, and a more thorough characterization of isolates may lead to the identification of bacterial factors that enhance the possibility for coinfection. Conversely, a small number of host factors have been identified that can alter the outcome of chlamydial infection (65) and differences in the outcome of both gonococcal and chlamydial infection in different mouse strains suggest that host genetic factors also play a role during infection with *N. gonorrhoeae* and *C. trachomatis* (63, 236). Therefore, host factors may play an important role in determining the susceptibility of an individual to coinfection.

The ultimate goal of research into the many different areas of uncertainty about gonococcal and chlamydial coinfection is the development of strategies designed to eliminate or prevent infection with both organisms simultaneously or prevent upper reproductive tract infection and tubal damage. In order to answer these many questions, however, experimental models are needed with which controlled experiments can be performed.

6.0 Goal and Specific Aims

The goal of this work was to develop and characterize a murine model of gonococcal and chlamydial coinfection in female mice.

6.1 Specific Aim 1

The first aim of this work was to **develop and characterize a female mouse model of gonococcal and chlamydial coinfection**. Female BALB/c mice were coinfected with *C. muridarum* and *N. gonorrhoeae* by establishing chlamydial infection first, in the absence of exogenous hormone treatment, and then challenging with *N. gonorrhoeae* following estradiol treatment to promote long-term gonococcal colonization. We found coinfection to be different from infection with either pathogen alone in two ways. First, mice coinfected with *C. muridarum* had a significant increase in the number of gonococci recovered compared to mice infected with *N. gonorrhoeae* alone as early as day one post-gonococcal challenge and this difference was maintained for several days. Additionally, coinfected mice had a greater PMN influx than uninfected mice or mice infected with either pathogen alone.

6.2 Specific Aim 2

The second aim of this work was to **determine the mechanism responsible for increased gonococcal colonization in *C. muridarum* coinfected mice**. Using a screen for changes in gene expression in *C. muridarum*-infected compared to uninfected mice prior to challenge with *N. gonorrhoeae*, we found levels of transcript for two AMPs, cathelicidin related antimicrobial peptide (CRAMP) and serine leukocyte peptidase inhibitor (SLPI), to be decreased. Additionally, levels of transcript for all TLRs examined were upregulated in *C. muridarum*-infected mice compared to uninfected mice, except TLR4, which was previously shown by our lab to be protective during gonococcal infection (237). The role of TLR4 was confirmed using flow cytometry and knock-out mice. *C. muridarum* infection resulted in a decrease in the percentage of TLR4-positive

genital epithelial cells prior to gonococcal challenge and this is likely responsible for the increase in gonococcal colonization.

Chapter 2: Chlamydial Infection Increases Gonococcal Colonization in a Novel Murine Coinfection Model

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The work presented in this chapter is the sole work of R.A. Vonck, with the following exceptions: A.E. Jerse advised all experimentation and writing and A.E. Jerse, T. Darville, and C.M. O'Connell assisted with experimental design and editing of the manuscript.

Abstract

Genital tract infections caused by *Neisseria gonorrhoeae* and *Chlamydia trachomatis* serovars D-K occur at high incidence in many areas of the world. Despite high rates of coinfection with these pathogens, investigations of host-parasite interactions have focused on each pathogen individually. Here we describe a coinfection model in which female BALB/c mice were first infected with the mouse *Chlamydia* species *C. muridarum* and then inoculated with *N. gonorrhoeae* following treatment with water-soluble 17β-estradiol to promote long-term gonococcal infection. Viable gonococci and chlamydiae were recovered for an average of 8-10 days, and diplococci and chlamydial inclusions were observed in lower genital tract tissue by immunohistochemical staining. Estradiol treatment reduced proinflammatory cytokine and chemokine levels in

chlamydiae-infected mice; however, coinfected mice had a higher percentage of vaginal neutrophils compared to mice infected with either pathogen alone. We detected no difference in pathogen-specific antibody levels due to coinfection. Interestingly, significantly more gonococci were recovered from coinfected mice compared to mice infected with *N. gonorrhoeae* alone. We found no evidence that *C. muridarum* increases gonococcal adherence to, or invasion of immortalized murine epithelial cells. However, increased vaginal concentrations of inflammatory mediators MIP-2 and TNF-α were detected in *C. muridarum*-infected mice prior to inoculation with *N. gonorrhoeae* concurrently with down-regulation of cathelicidin-related antimicrobial peptide (CRAMP) and secretory leukocyte peptidase inhibitor (SLPI) genes. We conclude that female mice can be successfully infected with both *C. muridarum* and *N. gonorrhoeae*, and that chlamydiae-induced alterations in host innate responses may enhance gonococcal infection.

Introduction

Chlamydia and gonorrhea are the two most common notifiable infectious diseases in the United States with over 1 million cases of chlamydia and 350,000 cases of gonorrhea reported to the Centers for Disease Control in 2008 (44). Actual rates of infection are much higher due to high rates of asymptomatic infection (199). As many as 50-70% of individuals with gonorrhea also have a chlamydial infection (72, 199, 227), and empirical treatment for chlamydia upon detection of *N. gonorrhoeae* is recommended (47, 104). *Neisseria gonorrhoeae* and *Chlamydia trachomatis* are both Gram-negative, human-specific pathogens. In symptomatic infections, both organisms elicit a

proinflammatory response characterized by the influx of polymorphonuclear leukocytes (PMNs). Clinically, gonorrhea is typically more pyogenic. Post-infection complications can occur with either pathogen and complications are generally more common and more severe in women. Infections that ascend to the upper genital tract in women lead to pelvic inflammatory disease (PID), the complications of which include chronic pelvic pain, ectopic pregnancy, and infertility (136, 321).

The incidence (199, 227), transmission (179, 181, 188), and associated symptoms and complications (282, 285) of gonococcal and chlamydial coinfection have been examined in epidemiological studies. However, differences in the pathogenesis and host response to coinfection have not been investigated in an infection model. *N. gonorrhoeae* is primarily extracellular, but can invade and replicate within epithelial cells (71, 79). While the inflammatory response to *N. gonorrhoeae* can be robust in symptomatic infections, gonococcal infection induces only a transient antibody response (317). Recent evidence suggests that IL-17 responses are induced during gonococcal infection and that both IL-17 (88) and toll-like receptor 4 (TLR4) are protective (237). In contrast, *C. trachomatis* is an obligate, intracellular parasite that undergoes a complex life cycle within the host cell involving the infectious, metabolically inactive elementary body (EB) and the metabolically active, replicative reticulate body (RB) (1). The primary immune response to *C. trachomatis* is through the Th1 pathway (63).

Despite these differences in life-styles and host response, how one organism may alter the pathogenesis, disease severity, susceptibility and host response to the other pathogen is not known. A small animal model of gonococcal and chlamydial coinfection is needed to facilitate the investigation of aspects of pathogenesis that are unique to

coinfection and the development of improved prophylactic products. Well-established female mouse models of gonococcal or chlamydial infection currently exist. The development of a coinfection model, however, is challenged by differences in the susceptibility of mice to each pathogen with respect to the stage of the reproductive cycle. The mouse model of gonococcal genital tract infection capitalizes on the transient susceptibility of female mice to *N. gonorrhoeae* that occurs during the proestrus stage of the estrous cycle (62, 152). In this model, mice are treated with 17β-estradiol and antibiotics to promote long-term colonization with *N. gonorrhoeae* (144). In the most recent modification of this model, water-soluble estradiol is used, and serum estradiol concentrations return to physiological levels by day 3 post-infection and mice are colonized for 10-12 days (317). Gonococci are detected within murine vaginal and cervical tissue (317) and ascend to the upper genital tract in approximately 18-20% of mice (144). Infected mice have an inflammatory response that is characterized by PMN influx and the induction of TNFα, IL-6, MIP-2, and KC (144, 236, 315), and similar to human infection, mice elicit an unremarkable humoral response, and are susceptible to repeat infection with the same strain (317). Female mice are most susceptible to *Chlamydia* species when in the progesterone-dominant phase of the reproductive cycle and protocols for infection with *Chlamydia muridarum* (formerly *C. trachomatis* MoPn) (15) or human serovars of *C. trachomatis* that use or do not use progesterone treatment have been developed (15, 21, 270). The course of infection with the mouse pneumonitis agent, *C. muridarum*, appears more similar to human genital chlamydial disease than infection of mice with human serovars of *C. trachomatis*. *C. muridarum* ascends from the lower to the upper genital tract of female mice (15) and causes an inflammatory

response that is characterized by infiltration of both acute and chronic inflammatory cells, and post-infection sequelae, such as tubal occlusion, hydrosalpinx, and infertility (63, 69).

Here we describe the successful coinfection of female mice with *N. gonorrhoeae* and *C. muridarum*. Differences in colonization load and the host immune response occurred in coinfected mice compared to mice infected with either pathogen alone. This model should serve as a useful research tool for further study of gonococcal and chlamydial coinfection and the development of prophylactic and therapeutic agents against bacterial cervicitis and PID.

Materials and Methods

Bacterial propagation

N. gonorrhoeae strain FA1090 [*por*B1b, AHU (an auxotype for arginine, hypoxanthine, and uracil), serum resistant] was originally isolated from a female with disseminated gonococcal infection (54). Frozen stocks of piliated, OpaB-expressing FA1090 bacteria isolated from a male volunteer (145) were passaged on solid GC agar containing Kellogg's supplement I (161) and 12 μM $Fe(NO_3)_3$ and incubated at 37°C in a humidified 7% CO_2 incubator. GC agar with antibiotic selection [GC-vancomycin, colistin, nystatin, trimethoprim sulfate, and streptomycin (VCNTS)] and heart infusion agar (HIA) were used to isolate *N. gonorrhoeae* and facultatively anaerobic commensal flora, respectively, from murine vaginal mucus as described (144). *C. muridarum* strain Nigg (289) was propagated in L929 mouse fibroblast cells (gift of Dr. Anthony T. Maurelli, Uniformed Services University, Bethesda, MD) and *C. trachomatis* serovar D

(gift of Dr. Anthony T. Maurelli) was propagated in ME180 human cervical epithelial cells, similarly to the method described by O'Connell and Nicks (231). Briefly, monolayers of L929 or ME180 cells in 24-well plates were inoculated with *C. muridarum* or *C. trachomatis*, respectively, suspended in infection media [1X Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 µg/ml gentamicin, and 100 µg/ml cycloheximide] at a multiplicity of infection (MOI) of 1. Plates were centrifuged at 1600 x *g* for 1 hr at 37°C and media was replaced with fresh infection media. Infected monolayers were incubated at 37°C, 5% CO_2 for 36 hrs before being harvested into SPG buffer (218 mM sucrose, 3.8 mM KH_2PO_4, 7.2 mM K_2HPO_4, 4.9 mM L-glutamate, pH 7.2), sonicated briefly, and frozen at -80°C. The titer of inclusion forming units (IFU) in each stock was determined by immunofluorescence as described by Kelly *et.al.* (165) except that inclusions were stained with ChlamIII anti-chlamydial LPS antibody (Santa Cruz Biotechnology) at a 1:500 dilution and goat anti-mouse AlexaFlour-488 secondary antibody (Invitrogen) at a 1:2000 dilution. L929 mouse fibroblast cells were maintained in DMEM with 10% FBS and grown to large quantities in suspension culture in RPMI with 5% FBS. ME180 human cervical epithelial cells were maintained in McCoy's 5A media supplemented with 10% FBS and 2.2 g/L $NaHCO_3$. Solid agar, cell culture reagents, and chemicals were purchased from Difco, Quality Biological, and Sigma, respectively, unless otherwise noted.

Coinfection protocol

Female BALB/c mice 4-6 weeks in age were purchased from the National Cancer Institute (Bethesda, MD). The infection protocol is shown in Figure 1. Except when noted, each experiment consisted of four groups of mice: mice coinfected with *N.*

gonorrhoeae and *C. muridarum*, mice infected with either pathogen alone, and mice inoculated with buffers alone as a control for inflammation (n = 10-11 mice per group in each of three experiments). Inoculations with *C. muridarum* were performed on anesthetized mice for the purpose of immobilization by intraperitoneal (i.p.) injection of a ketamine/xylazine mixture (10 mg and 1.5 mg, respectively, per 100 g body weight). Mice were then vaginally inoculated with $3x10^5$ IFU of *C. muridarum* in 20 µl of 2-SP buffer (200 mM sucrose, 12 mM K_2HPO_4, 8 mM KH_2PO_4) by pipette on three consecutive days to increase the likelihood that mice were in the diestrus stage of the reproductive cycle (270). The dose of *C. muridarum* was calculated based on the titer of the frozen stock determined as described above. Mice infected with *N. gonorrhoeae* alone and control mice were similarly anesthetized and mock-inoculated with 10 µl of 2-SP buffer. On the final day of *C. muridarum* inoculation, vaginal smears from all mice were prepared on glass slides and stained with a Hema-3 stain (Fisher Scientific) to identify mice in the diestrus stage as described (62). Mice with a predominance of PMNs and nucleated epithelial cells, rather than squamous epithelial cells, were considered to be in diestrus. Mice found to be in diestrus were then treated with a subcutaneous injection of 0.5 mg of water-soluble 17β-estradiol ($estradiol_{ws}$, Sigma) (317) approximately six hrs following the final inoculation with *C. muridarum* or buffer, and 2 and 4 days later. Four hrs after the second dose of $estradiol_{ws}$, mice were vaginally inoculated with $1x10^6$ colony forming units (CFU) of *N. gonorrhoeae* in 20 µl of phosphate buffered saline (PBS) (*N. gonorrhoeae* only and coinfected groups), or mock-inoculated with PBS (*C. muridarum* only and uninfected control groups). The *N. gonorrhoeae* inoculum was prepared as described elsewhere (144) and the dose confirmed by quantitative culture.

All mice were given vancomycin hydrochloride (0.6 mg, twice daily) and streptomycin sulfate (2.4 mg, twice daily) via i.p. injection beginning two days before *N. gonorrhoeae* inoculation and maintained for seven days to control overgrowth of commensal flora. In two experiments, *C. muridarum* infection was allowed to occur for 8-10 days prior to treatment with estradiol$_{ws}$ and inoculation with *N. gonorrhoeae*. In these experiments, n = 5 and 10 and the combined results are reported.

Quantitation of colonization load and PMN influx

The number of gonococci and chlamydiae recovered from each group was determined daily for 10 days in three separate experiments consisting of four groups (n = 10-11 mice per group in each experiment). Vaginal mucus was collected with a PBS-soaked polyester swab and a small portion of the sample was inoculated onto an HIA plate for recovery of facultatively anaerobic commensal flora and a glass slide for enumeration of PMNs. The percentage of PMNs per 100 vaginal cells was determined by cytological differentiation of stained vaginal cells as described (62). The remaining sample was then suspended in 1 ml of transport buffer (2-SP buffer supplemented with 3% FBS and 0.5 mg/ml vancomycin). Suspensions were cultured onto GC-VCNTS agar to isolate *N. gonorrhoeae* using the Autoplater 4000 (Spiral Biotech) and then frozen at -80°C for culture of *C. muridarum*. The number of CFU of *N. gonorrhoeae* recovered was enumerated using the Spiral Biotech Q-Counter Software. For *C. muridarum*, suspensions were diluted and cultured onto monolayers of L929 cells and quantified by immunofluorescence as described above. The limits of detection were 20 CFU (*N. gonorrhoeae*) and 12.5 IFU (*C. muridarum*) per ml vaginal swab suspension. Upper genital tracts (uterine horns, oviducts, and ovaries) were cultured for *N. gonorrhoeae* and

C. muridarum 10 days after inoculation with *N. gonorrhoeae* or PBS (chlamydia alone group) in a single experiment consisting of 12 mice per group. Upper genital tracts were homogenized in 2-SP buffer. The homogenate was serially diluted in PBS and cultured for *N. gonorrhoeae* on GC-VCNTS. The remaining homogenate was frozen at -80°C and *C. muridarum* titers were determined using a plaque assay (231) except that monolayers of L929 cells were used.

Enzyme-linked immunosorbent assay (ELISA) for C. muridarum- *and* N. gonorrhoeae-*specific antibodies*

Vaginal washes and sera were collected on days 10 and 28 post-challenge with *N. gonorrhoeae* (*N. gonorrhoeae*–infected and coinfected groups) or PBS (uninfected and *C. muridarum*-infected groups) in the 3 experiments performed to assess gonococcal and chlamydial colonization and examined for chlamydia- or gonococcal-specific antibodies. EBs from *C. muridarum* strain Nigg were purified by Renograffin (Bracco Diagnostics) gradient (41) and outer membrane vesicles (OMVs) from *N. gonorrhoeae* strain FA1090 were prepared as described (30). Ninety-six well plates were coated with 50 µl of either *C. muridarum* EBs (5 µg/ml) or *N. gonorrhoeae* OMVs (10 µg/ml) in 0.5 M $NaHCO_3$ overnight at room temperature. All washes were performed with PBS containing 0.1% Tween-20 using the Molecular Devices Skan Washer. Wells were blocked with PBS containing 15% FBS for 30 min at 37°C in a humidified chamber. Sera were diluted 1:100 and 1:900 and vaginal washes were diluted 1:30 and 1:100 in the block solution and added to the wells (50 µl) followed by incubation with secondary antibody (goat anti-mouse IgG, IgM, or IgA conjugated to horse radish peroxidase, Sigma) diluted 1:10,000 in the block solution. Incubations with primary and secondary antibodies were for one hr

at 37°C in a humidified chamber. TMB-peroxidase Detection Solution (BioRad) was added to detect bound secondary antibody. The reaction was stopped after ten min with 0.1 N H_2SO_4 and the optical density at 450 nm was read using Biotek Instruments EL80 Universal Microplate Reader and KC Junior software.

Cytokine and chemokine protein analysis

Genital tract secretions were collected for cytokine/chemokine analysis in a single experiment consisting of the following five groups: estradiol-treated mice that were infected with either single agent or both agents or left uninfected and mice infected with *C. muridarum* and not treated with estradiol (n = 4-5 mice/group). Genital tract secretions were collected with absorbent sponges (DeRoyal Earwick) and proteins were eluted as described (64). Levels of IFNγ, IL-1β, IL-10, MIP-2, RANTES, TNF-α, and IL-17 protein were measured using the Millipore Mouse Cytokine/Chemokine Milliplex^{TM} Map Kit as instructed. Reactions were read on the Luminex 100^{TM} IS instrument and mean fluorescent intensity (MFI) was compared to standard curves to calculate pg/ml concentrations of each protein using the Luminex 100^{TM} IS software.

Immunohistochemical tissue analysis

Tissue was collected for histological examination during a single experiment consisting of coinfected mice, mice infected with either pathogen alone, and uninfected control mice. Whole genital tracts were harvested from 5 mice per group on day 2 post-inoculation with *N. gonorrhoeae* or PBS (chlamydia alone), and tissue was fixed in 10% buffered formalin for 24 hrs and then stored in 70% ethanol prior to embedding in paraffin and sectioning onto slides for immunohistochemical analysis. Sections were double immunolabeled for *C. muridarum* using the Chlam-III anti-chlamydial antibody

(Santa Cruz Biotechnology) at a 1:50 dilution and mouse anti-serum raised against *N. gonorrhoeae* strain FA1090 outer membrane vesicles at a 1:500 dilution by Histoserv, Inc. (Germantown, MD).

Tissue culture adherence assay

ME180 human cervical epithelial cells were cultured as described above. IEC4.1 mouse intestinal epithelial and BM1.11 mouse oviduct cells [gifts of Drs. Harlan Caldwell (Rocky Mountain Laboratories, Hamilton, MT) and Raymond Johnson (Indiana University School of Medicine, Indianapolis, IN), respectively] were cultured as described elsewhere (286). Cells were seeded into 24-well plates and cultured to obtain monolayers of 80-90% confluency, and inoculated with *C. trachomatis* (ME180 cells) or *C. muridarum* (IEC4.1 and BM11.1 cells) at an MOI of 1 or mock-inoculated with SPG buffer with centrifugation in culture media, as described above. After 20 hr incubation in normal culture medium, *N. gonorrhoeae* strain FA1090 was diluted in PBS to an OD_{600} of 0.07, followed by dilution in RPMI (Quality Biological) supplemented with 10% FBS and 0.3 μM $Fe(NO_3)_3$, and monolayers were inoculated with 500 μl of the *N. gonorrhoeae* suspension (final MOI = 1). After 2 hrs at 37°C in 7% CO_2, the number of cell-associated gonococci was determined by washing monolayers 4 times with PBS followed by host cell lysis with 0.5% saponin in PBS and quantitative culture. Invasion was measured by the gentamicin (Gm) protection assay. For intracellular bacteria, monolayers were washed 2 times with PBS after 2 hrs incubation, and 500 μl RPMI supplemented with 10% FBS, 0.3 μM $Fe(NO_3)_3$, and 50 μg/ml Gm was added for 1.5 hours. Monolayers were washed 5 times with PBS and cells were lysed in 0.5% saponin in PBS followed by quantitative culture. Results are expressed as the percent of cell-

associated bacteria relative to the inocula (% adherence) or as the percent of Gm-protected bacteria relative to the number of adherent bacteria under the same conditions (% invasion). Conditions were performed in triplicate and each experiment was repeated at least three times.

Gene expression analysis by RT-PCR

Vaginal material was collected with a PBS-soaked polyester swab and suspended directly in 500 µl RNA-Later (Ambion) from uninfected or *C. muridarum*-infected mice 15 min prior to inoculation with *N. gonorrhoeae* to determine expression levels of the cathelicidin-related antimicrobial peptide (CRAMP) gene *cnlp*, and the secretory leukocyte peptidase inhibitor (SLPI) gene. Samples were stored at -80°C until use. Total RNA was extracted using the Qiagen mini-RNeasy isolation kit per the manufacturer's instructions. RNA was converted to cDNA using the SABioscience RT^2 EZ First Strand cDNA kit and then used for real-time PCR. cDNA reaction mixtures (20 µl total volume) were diluted to a final volume of 100 µl in nuclease-free water and 5 µl of diluted cDNA template was subjected to PCR amplification using an ABI 7500 sequence detector [25 µl total reaction volume consisting of template, 12.5 µl SYBR green master mix (ABI), and 10 µl primer mix, which contained both forward and reverse primers at concentrations of 1 µM]. Reactions were performed according to the following parameters: 10 min at 95°C, then 40 cycles at 95°C for 15 s and at 60°C for 1 min. The cycle threshold (C_T) value was determined using the Sequence Detector v.1.7a software (ABI). Data were analyzed with Microsoft Excel using the comparative C_T method ($\Delta\Delta C_T$) for relative quantification of gene expression using β-actin as the normalizer, as described (236). Expression of the CRAMP and SLPI genes was measured by normalizing to the β-actin

gene. The calculation used included the difference between the C_T values of the normalizer (β-actin) and the C_T values of the test genes (*cnlp* and SLPI) in individual samples, as follows: $\Delta C_{T(\textit{C. muridarum} \text{ infected or uninfected})} = C_{T(\beta\text{-actin})} - C_{T(\text{test gene})}$. ΔC_T values for all uninfected mice were calculated and the mean of these values was used as a baseline for calculating the $\Delta\Delta C_T$ value for each *C. muridarum*-infected mouse. The relative difference in gene expression between each *C. muridarum*-infected mouse and the uninfected baseline value on day 0 (prior to inoculation with *N. gonorrhoeae*) was defined as the difference of normalized gene expression levels as follows: $2^{-\Delta\Delta C_T}$ where $\Delta\Delta C_T = \Delta C_{T(\text{uninfected baseline})} - \Delta C_{T(\text{infected})}$. The experiment was performed twice with 4-5 mice per group. The sequences of the oligonucleotide primers are shown in Table I.

Statistical analysis

Differences in gonococcal and chlamydial colonization load between coinfected and single-pathogen infected groups were assessed by repeated measures ANOVA. PMN influx was compared between all groups by ANOVA using the Bonferroni correction for multiple comparisons. Unpaired t-tests were performed on results from individual days. Levels of gonococcal cell-association and invasion were compared by unpaired t-tests, as were cytokine and chemokine levels in genital tract secretions. P-values of ≤ 0.05 were considered significant.

Animal use assurances

All animal experiments were conducted in the laboratory animal facility at the Uniformed Services University, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, under a protocol approved by the university's Institutional Animal Care and Use Committee.

Table 1. Oligonucleotide sequences used in this study

Gene	Primer Sequence (5'-3') Forward	Reverse	Source
CRAMP	AGGAGATCTTGGGAACCATGCAGTT	GCAGATCTACTGTCCGGCTGAGGTA	(218)
SLPI	CGGCTCTGGACTCGTGCTCGG	GCAATAAGTGGCCGTGGTGTG	(314)
β-actin	GCGCAAGTACTCTGTGTGGA	CATCGTACTCCTGCTTGCTG	(236)

Results

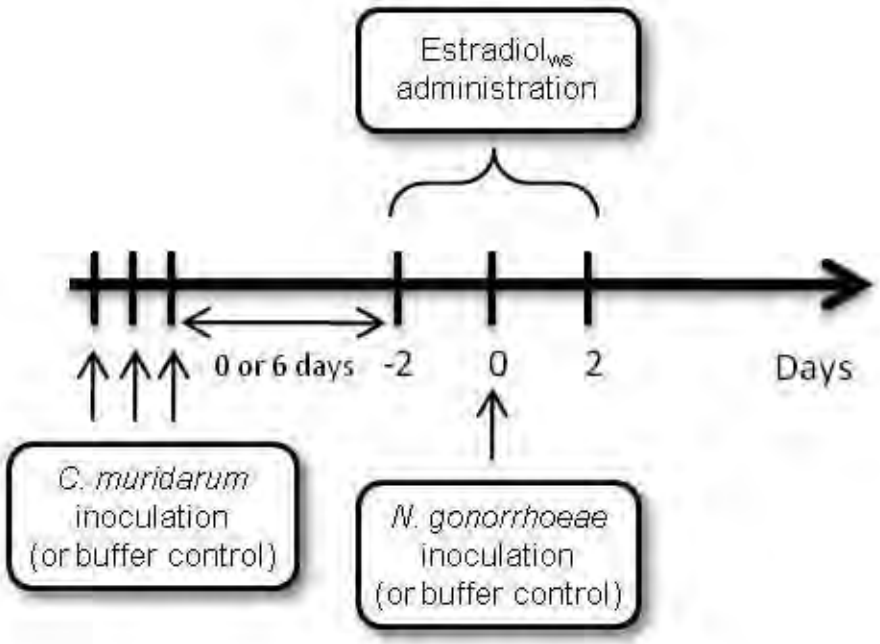
Coinfection of female mice with N. gonorrhoeae and C. muridarum

Female mice are most susceptible to infection with *N. gonorrhoeae* during the estrogen-dominant stage of the estrous cycle (62, 152, 169) and *C. muridarum* during the progesterone-dominant stage of the estrous cycle (21, 338). Progesterone treatment is not required for long term colonization with *C. muridarum* provided that mice are inoculated during the diestrus stage of the estrous cycle (270). In contrast, susceptibility to long term colonization with *N. gonorrhoeae* requires administration of estradiol to promote an estrus-like state. In pilot experiments, our attempts to coinfect estradiol-treated female BALB/c mice with *N. gonorrhoeae* and *C. muridarum* simultaneously were unsuccessful. However, we found that once *C. muridarum* infection was established, subsequent treatment with estradiol did not cause chlamydial infection to clear or alter the number of chlamydial inclusions recovered, in agreement with previous observations (163). Thus, we developed a model in which chlamydial infection was established followed by inoculation with *N. gonorrhoeae* (Figure 7).

Figure 7. Time line for coinfection protocol.

Mice were inoculated with *C. muridarum* on three consecutive days to establish chlamydial infection. Following the final inoculation with *C. muridarum*, mice in the diestrus stage of the estrous cycle were treated with water-soluble estradiol and inoculated with a single dose of *N. gonorrhoeae* two days later. In the experiments described, mice were infected with *C. muridarum* for either 2-4 days or 8-10 days prior to inoculation with *N. gonorrhoeae*.

Figure 7. Time line for coinfection protocol.



Using this protocol viable gonococci and chlamydiae were recovered from vaginal swabs for an average of 6-7 days and 9-10 days, respectively, post inoculation with *N. gonorrhoeae* in three separate experiments (Tables II and III). *N. gonorrhoeae* diplococci and *C. muridarum* inclusions were visualized in genital tract tissues of coinfected mice by immunohistochemical staining (Figure 8). *C. muridarum* inclusions were restricted to the cervix of coinfected animals. Gonococci were observed in the lumens of the vagina and the cervix as well as deep within the vaginal tissue at two days post-gonococcal challenge. We conclude that coinfection of female mice with *N. gonorrhoeae* and *C. muridarum* can be established.

We saw no difference in the rate of ascendant infection with either pathogen. Ascendant chlamydial infection was observed in 75% of *C. muridarum*-infected mice and this percentage was not altered by coinfection with *N. gonorrhoeae* (mean of 10^3 PFU of *C. muridarum* per upper genital tract in both groups; data not shown). This rate of ascension is comparable to rates seen in progesterone-treated C57/BL6 mice (229). No gonococci were recovered from the upper genital tract at day 10 post-gonococcal challenge in mice with or without a pre-existing chlamydial infection in these experiments.

Table 2. Coinfection does not alter the duration of recovery of N. gonorrhoeae

	Mean Duration of Bacterial Recovery (Range)[a]	
	***N. gonorrhoeae* Alone**	**Coinfected**
Experiment I	6.4 (1-10)	8.0 (4-10)
Experiment II	6.1 (2-9)	6.1 (4-9)
Experiment III	7.6 (1-10)	9.7 (7-10)
Cumulative	**6.6 (1-10)**	**7.7 (4-10)**

[a]Values represent the mean number of days out of 10 consecutive days *N. gonorrhoeae* was recovered by vaginal swab from mice in three separate experiments with the range in days represented in parenthesis.

Table 3. Coinfection does not alter the duration of recovery of C. muridarum

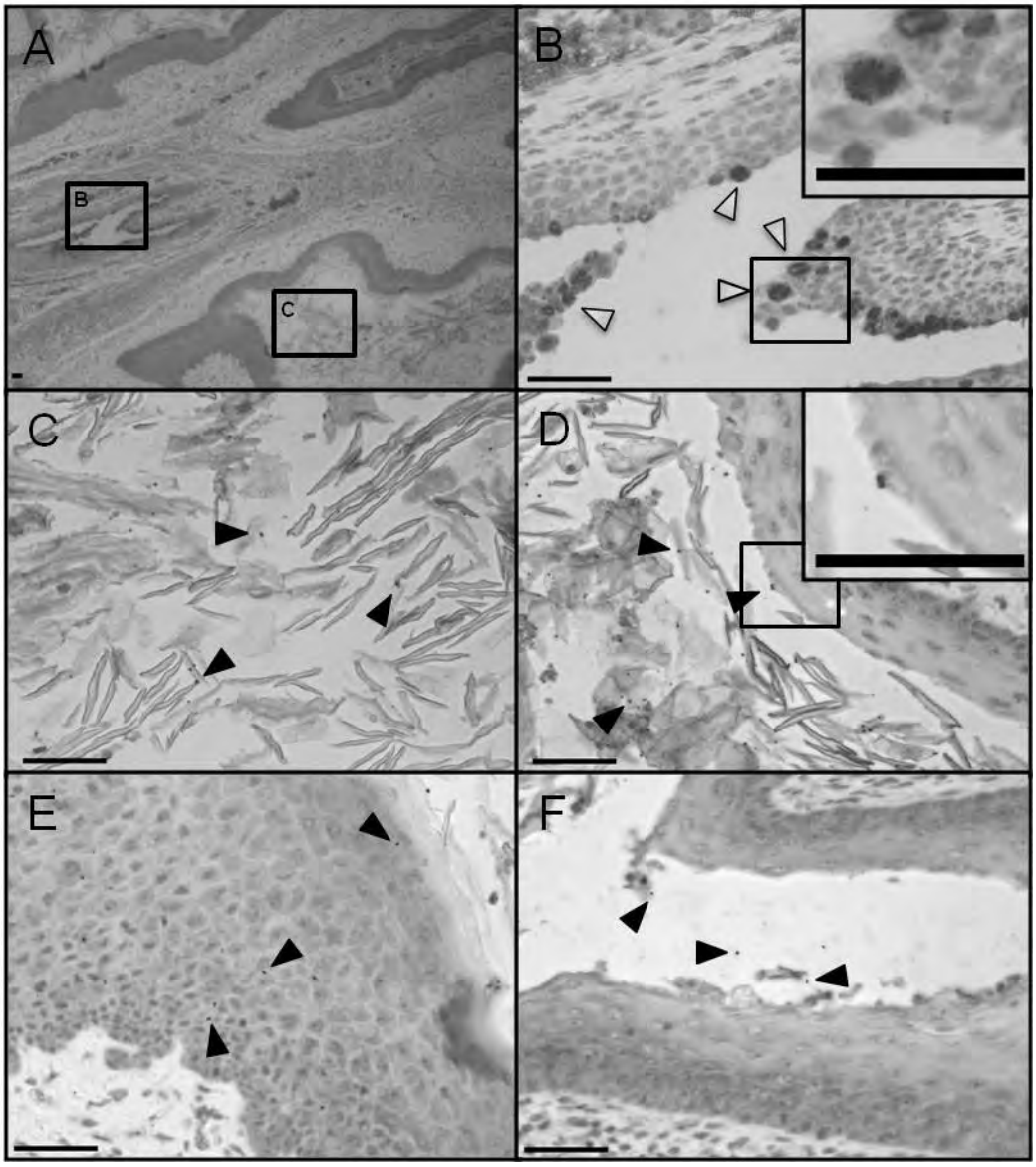
	Mean Duration of Bacterial Recovery (Range)[a]	
	***C. muridarum* Alone**	**Coinfected**
Experiment I	9.2 (8-10)	9.5 (8-10)
Experiment II	9.9 (9-10)	9.8 (9-10)
Experiment III	9.0 (5-10)	8.0 (5-10)
Cumulative	**9.5 (5-10)**	**9.2 (5-10)**

[a]Values represent the mean number of days out of 10 consecutive days *C. muridarum* was recovered by vaginal swab from mice in three separate experiments with the range in days represented in parenthesis.

Figure 8. Immunohistochemical staining reveals the presence of N. gonorrhoeae and C. muridarum in the coinfected genital tract.

(A) Genital tract tissue extracted from a coinfected mouse on day 2 post-*N. gonorrhoeae* inoculation at 40X magnification. Boxes labeled B and C correspond to the regions shown in panels B and C, respectively, where they are magnified at 400X. (B) Cervical tissue at 400X magnification showing distinct *C. muridarum* inclusions (gray arrows) within nucleated epithelial cells. Panels C, D, E, and F show tissue from the same mouse at 400X magnification with visible *N. gonorrhoeae* diplococci (black arrows) among squamous epithelial cells in the vaginal lumen (C), squamous epithelial cells in the vaginal lumen and superficially associated with the vaginal epithelium (D), deep within the vaginal tissue (E), and in the lumen of the cervix (F). Several chlamydial inclusions and a single diplococcus are shown in the insets of higher magnification in panels B and D, respectively. All scale bars represent 50 μm. The dual staining performed by Histoserv, Inc. was done with a different color chromagen for each organism for positive identification.

Figure 8. Immunohistochemical staining reveals the presence of N. gonorrhoeae and C. muridarum in the coinfected genital tract.



Estradiol treatment alters the host response to C. muridarum

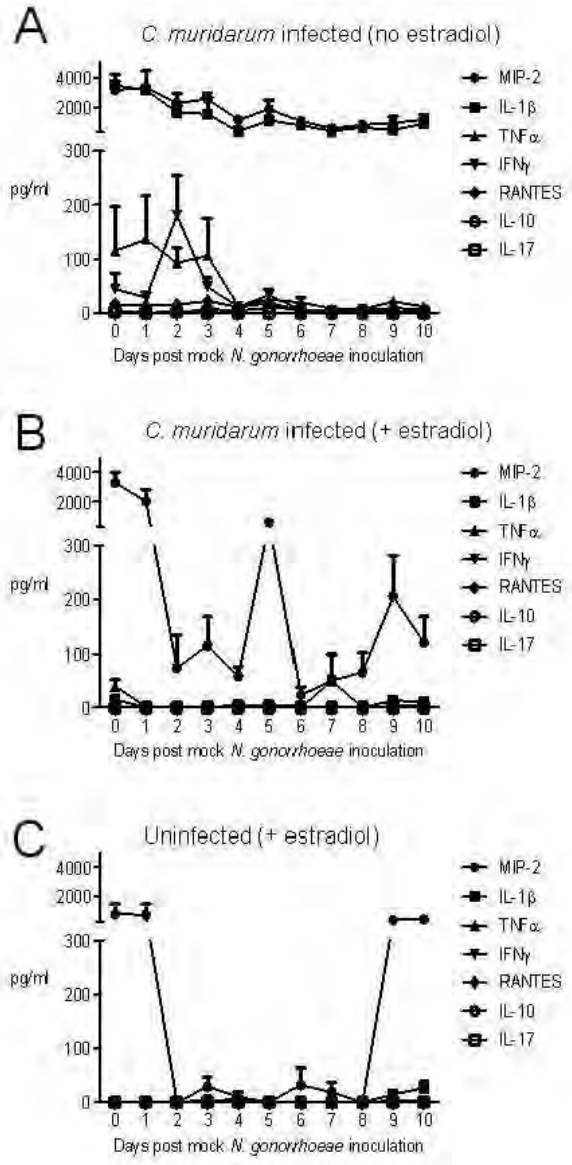
The initial inflammatory response to *C. muridarum* infection in progesterone-treated mice is intense and wanes over time (63). We defined the kinetics of the inflammatory response to *C. muridarum* in our model system by investigating the effects of estradiol on the inflammatory response to *C. muridarum* alone in the absence of inoculation with *N. gonorrhoeae*. Mice were infected with *C. muridarum* or mock-inoculated with buffer (uninfected control) on three consecutive days. On the final day of *C. muridarum* inoculation, 10 diestrus-stage mice were identified and half of these mice were treated with estradiol, as per the usual protocol, and half were not (n = 5 mice per group). All diestrus-stage mice in the uninfected control group were treated with estradiol (n = 4 mice). Two days later, all mice were inoculated with PBS, rather than *N. gonorrhoeae*. Vaginal levels of seven different cytokines and chemokines (MIP-2, IL-1β, TNF-α, IFN-γ, RANTES, IL-10, and IL-17) that were previously implicated in gonococcal (53, 77, 88, 185, 236) or chlamydial infection (20, 63, 64, 273, 281, 363) were measured on the day of PBS challenge and over the next 10 days. Consistent with *C. muridarum* inducing an inflammatory response, high levels of IFN-γ, TNF-α, IL-1β, and MIP-2 were detected in mice that were not treated with estradiol on days 1-4 post-inoculation. MIP-2 and IL-1β levels remained elevated over the next 7 days, while IFN-γ and TNF-α levels declined at a time point that corresponds to day 4 post-inoculation with *N. gonorrhoeae* in our coinfection protocol (Figure 9A). Administration of estradiol dampened the response to *C. muridarum* as evidenced by similar levels of IL-1β, TNFα, and IFNγ in estradiol-treated, infected (Figure 9B) and estradiol-treated, uninfected (Figure 9C) mice. Interestingly, estradiol treatment did not fully abrogate the MIP-2

response to *C. muridarum* as vaginal MIP-2 levels were elevated in estradiol-treated, infected mice compared to uninfected, estradiol-treated mice on days 2-8 post-PBS challenge (Figure 9C). The high levels of MIP-2 in estradiol-treated, uninfected mice on days 0 and 1 and at late time points (days 9 and 10) when the effects of estradiol begin to wear off reflect the normal fluctuations in MIP-2 that are associated with the influx of vaginal PMNs during the metestrus and diestrus phases of the estrous cycle (318). We conclude that the requirement to administer estradiol to establish gonococcal infection reduces the inflammatory response to chlamydial infection, but that significant levels of MIP-2 are still induced.

Figure 9. The host inflammatory response to C. muridarum infection is altered by estradiol treatment.

Protein concentrations (pg/ml) of cytokines and chemokines in genital tract secretions from (A) non-estradiol treated, *C. muridarum* infected mice, (B) estradiol-treated, *C. muridarum* infected mice, and (C) estradiol-treated, uninfected mice (n = 4-5 mice per group).

Figure 9. The host inflammatory response to C. muridarum infection is altered by estradiol treatment.



Vaginal PMN influx is increased in coinfected mice

An influx of PMNs is characteristic of symptomatic infection with *N. gonorrhoeae* or *C. trachomatis* in both women and men. Similarly, a localized PMN influx is observed in gonococcal infection of BALB/c mice (236, 317) and mouse models of chlamydial infection (64, 144, 238). To determine whether the PMN response is altered during coinfection, the percentages of PMNs in vaginal smears from estradiol-treated uninfected mice, coinfected mice, and mice infected with either *N. gonorrhoeae* or *C. muridarum* alone were compared. A similar percentage of PMNs was observed on stained vaginal smears from estradiol-treated mice infected with either *N. gonorrhoeae* or *C. muridarum* alone. In both groups, vaginal PMNs were detected beginning at day 5 post-challenge with *N. gonorrhoeae* (gonorrhea alone) or PBS (chlamydia alone group), and gradually increased with significantly higher percentages of PMNs detected in both groups on days 9 and 10 compared to uninfected mice (Fig. 10A, solid symbols comared to open circles). Vaginal PMNs were also observed in coinfected mice beginning on day 5 post-inoculation with *N. gonorrhoeae*, with significantly higher percentages of PMNs observed in coinfected mice versus uninfected mice on days 5-10. On days 6, 7, and 8 post-inoculation with *N. gonorrhoeae*, a significantly higher percentage of vaginal PMNs were detected in coinfected mice compared to mice infected with either pathogen alone (Figure 10A, open squares versus solid symbols).

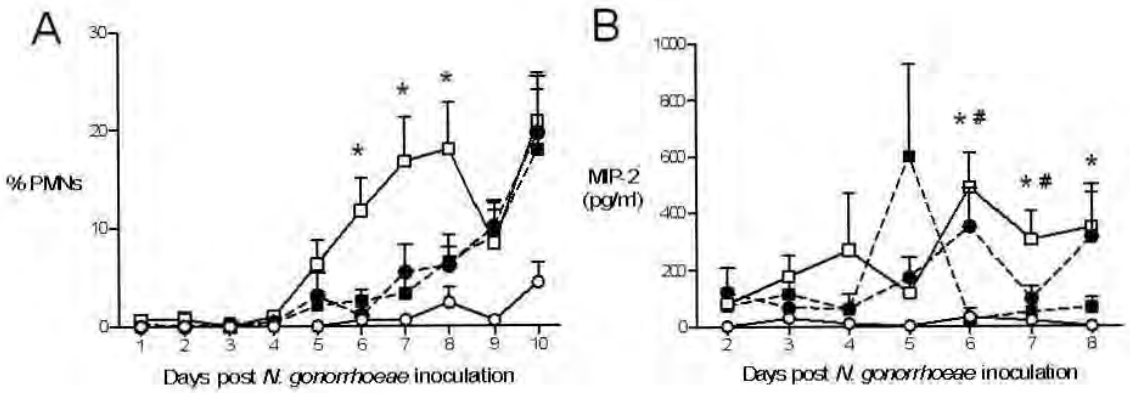
MIP-2, the mouse analog of the human PMN attracting chemokine IL-8, correlates with PMN influx at the infection site in both gonococcal and chlamydial mouse

infection models (64, 236). Here, levels of MIP-2 in genital tract secretions also mirrored the degree of vaginal PMN influx, with MIP-2 levels beginning to increase in coinfected mice and mice infected with either pathogen alone on day 5 and continuing to increase during the period of significantly increased PMN influx for all infected groups (days 6-8). MIP-2 levels, like the percentage of vaginal PMNs, were highest in the coinfected group on days 6, 7, and 8 post-inoculation with *N. gonorrhoeae* (Figure 10B). MIP-2 levels were elevated in *C. muridarum*-infected (similar to that shown in Figure 9B) and in coinfected mice, but not *N. gonorrhoeae*-infected mice on days 0 and 1, but decreased to below the limit of detection by day 2. After day 8, MIP-2 levels began to increase in all groups, including uninfected mice, as the effects of estradiol treatment wore off and mice began cycling again (data not shown). Other cytokines and chemokines tested (IL-1β, TNFα, IFNγ, RANTES, IL-10, and IL-17) were not significantly increased in coinfected mice at time points corresponding to changes in PMN influx (data not shown). In summary, these results suggest that despite the demonstrated immunosuppressive effect of the estradiol used in this model, an inflammatory response that results in a localized PMN influx is generated against mice infected with either single pathogen, and that this response is intensified in mice that are coinfected with *C. muridarum* and *N. gonorrhoeae*.

Figure 10. A greater PMN influx occurs in coinfected mice.

(A) Significantly more PMNs migrate into the lower genital tract of mice coinfected with *N. gonorrhoeae* and *C. muridarum* (white squares, solid line) compared to mice infected with *N. gonorrhoeae* (black circles, dashed line) or *C. muridarum* (black squares, dashed line) alone, or uninfected mice (white circles, solid line) on days 6, 7, and 8 post-*N. gonorrhoeae* inoculation (*$p < 0.05$). Percent PMNs is defined as (number of PMNs counted)/(100 vaginal cells observed, including squamous and nucleated epithelial cells and leukocytes). Results shown are from three combined experiments (n = 30 – 32 mice per group). (B) Levels of PMN attracting chemokine MIP-2 are greatest on days 6-8 in mice coinfected with *N. gonorrhoeae* and *C. muridarum* (white squares, solid line) compared to mice infected with either *N. gonorrhoeae* (black circles, dashed line) or *C. muridarum* (black squares, dashed line) alone or uninfected mice (white circles, solid line) on days 6-8 (*$p < 0.05$, coinfected vs. uninfected; # $p < 0.05$, coinfected vs. *C. muridarum* alone). Results from a single experiment are shown (n = 4 – 5 mice per group). The high average level of MIP-2 detected on day 5 in *C. muridarum*-infected mice is due to one mouse in this group having 1,843 pg/ml of MIP-2. MIP-2 concentrations ranged from 60-646 pg/ml for the other four mice within this group.

Figure 10. A greater PMN influx occurs in coinfected mice.



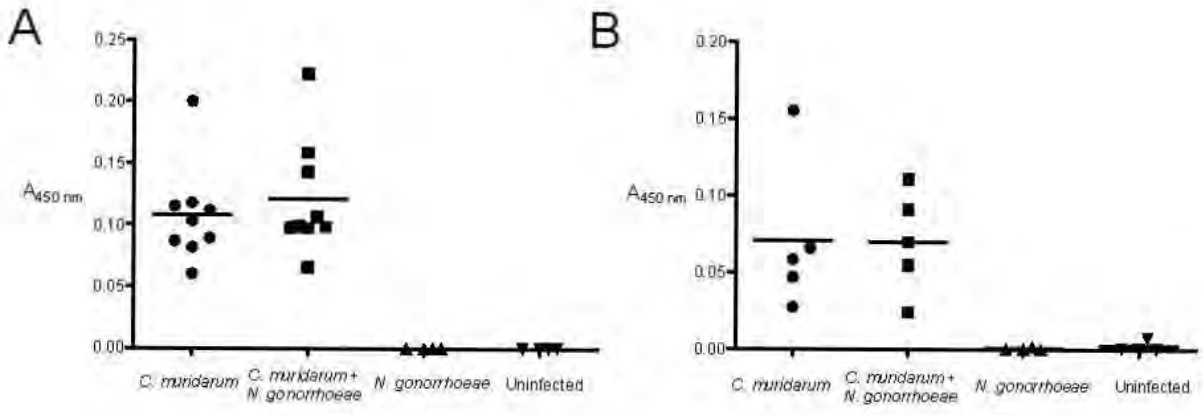
No difference was detected in the pathogen-specific antibody response

C. muridarum-infected mice respond robustly to infection and the antibody response peaks at approximately 4 weeks post-infection (15). In contrast, a weak antibody response occurs during *N. gonorrhoeae* murine infection, which is localized and transient and peaks at approximately 5 days post-infection (317). It is likely that increased or different interactions occur between innate receptors and bacterial ligands during coinfection compared to infection with either pathogen alone. We therefore hypothesized that coinfection may alter the humoral response to one or both pathogens. As expected, we detected significant levels of serum *C. muridarum*-specific IgG and IgM and vaginal IgA and IgG in chlamydia-infected mice on days 10 and 28. However, we found no difference between chlamydia-infected and coinfected mice (Figure 11 and data not shown). We were unable to detect gonococcal-specific antibodies in vaginal washes from coinfected mice or mice infected with *N. gonorrhoeae* alone on day 10 or 28 post-gonococcal challenge; earlier time points may have shown detectable antibodies (317). We conclude that while coinfection alters the innate immune response, there is no evidence that it affects the adaptive response at the level of antibody production.

Figure 11. Coinfection does not alter the level of C. muridarum-specific antibodies.

Levels of *C. muridarum*-specific serum IgG (A) and vaginal IgA (B) were measured by ELISA and the absorbance at 450 nm is shown for samples collected from mice infected with *C. muridarum* alone, mice coinfected with *C. muridarum* and *N. gonorrhoeae*, mice infected with *N. gonorrhoeae* alone, and uninfected mice in a single representative experiment. Samples were collected on day 28 post-inoculation with *N. gonorrhoeae*. Sera samples were diluted 1:900 and vaginal washes were diluted 1:100. Symbols indicate values for individual mice; horizontal bars show the geometric mean.

Figure 11. Coinfection does not alter the level of C. muridarum-specific antibodies.



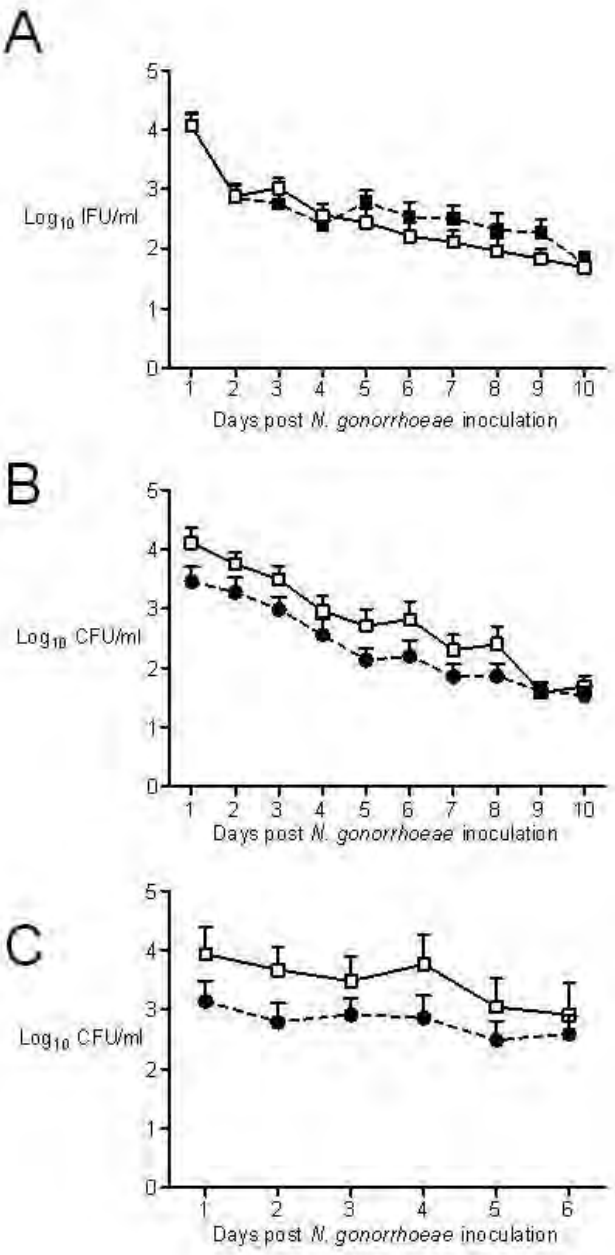
Gonococcal colonization is increased in coinfected mice

As discussed, we found no difference in the duration of infection with either pathogen in coinfected versus singly infected mice (Tables II and III). We also found no difference in the number of IFU of *C. muridarum* recovered from the lower genital tract of mice infected with *C. muridarum* alone or with both *C. muridarum* and *N. gonorrhoeae* (Figure 12A). However, a 0.5 - 1 log increase in the number of gonococci recovered from mice coinfected with *N. gonorrhoeae* and *C. muridarum* was observed as early as one day post-inoculation with *N. gonorrhoeae* compared to mice infected with *N. gonorrhoeae* alone (Figure 12B). This difference was maintained through approximately eight days post-inoculation with *N. gonorrhoeae* and was highly reproducible. To investigate whether an increased gonococcal colonization load would be seen in mice that were infected with *C. muridarum* for a longer time period before challenging with *N. gonorrhoeae*, we postponed estradiol treatment to 6 days after the final inoculation of *C. muridarum*. Mice were thus infected with *C. muridarum* for 8-10 days prior to challenge with *N. gonorrhoeae*, as described in Materials and Methods, which is when the initial intense inflammatory response to *C. muridarum* wanes and cytokine levels were the most similar between estradiol-treated and untreated *C. muridarum*-infected mice (Fig. 9). In two separate experiments we again observed increased gonococcal colonization in mice with a pre-existing *C. muridarum* infection (Figure 12C). We conclude that the effect of *C. muridarum* infection on gonococcal colonization is sustained during infection and can occur after the period in which the inflammatory response to *C. muridarum* peaks.

Figure 12. C. muridarum infection alters levels of N. gonorrhoeae in the lower genital tract.

(A) *C. muridarum* colonization remained constant whether mice were coinfected with *N. gonorrhoeae* (white squares, solid line) or infected with *C. muridarum* alone (black squares, dashed line) and (B) *N. gonorrhoeae* colonization was greater in mice coinfected with *C. muridarum* (white squares, solid line) than in mice infected with *N. gonorrhoeae* alone (black circle, dashed line) ($p = 0.011$ by repeated measures ANOVA). Results shown in panels A and B are from 3 combined experiments (n = 31-32 mice per group). (C) A similar trend in *N. gonorrhoeae* colonization during coinfection with *C. muridarum* was observed when mice with a longer pre-established *C. muridarum* infection were challenged with N. gonorrhoeae (n = 11-19 mice per group). The time point at which mice were challenged with *N. gonorrhoeae* in the experiment shown in panel C corresponds to day 6 in panels A and B. Results are expressed as $\log_{10}$ inclusion forming units (IFU) or colony forming units (CFU) per ml of vaginal swab suspension.

Figure 12. C. muridarum infection alters levels of N. gonorrhoeae in the lower genital tract.



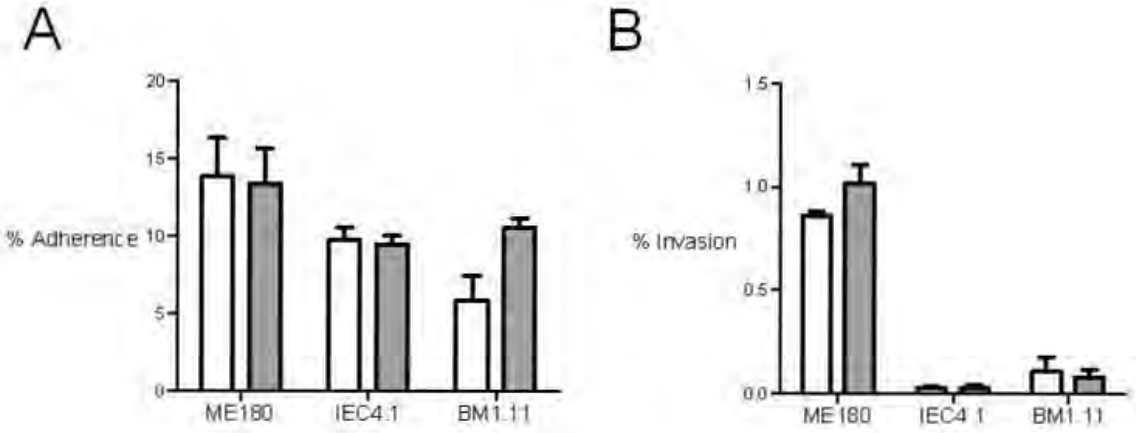
C. muridarum *does not affect* N. gonorrhoeae *association with cultured murine epithelial cells*

To investigate the mechanism behind the increased gonococcal colonization load in *C. muridarum* coinfected mice, we used an *in vitro* coinfection model to determine whether gonococci are more able to associate with and invade chlamydia-infected epithelial cells. We hypothesized that chlamydial infection may alter the epithelial cell surface to allow greater gonococcal adherence or invasion, thus accounting for the increased gonococcal colonization load observed *in vivo*. Murine epithelial cell lines of intestinal and oviduct origin were infected with *C. muridarum* or left uninfected and incubated for 20 hrs prior to inoculation with *N. gonorrhoeae*, which is the time point that coincides with active RB replication and the peak epithelial inflammatory cytokine response to chlamydial infection observed *in vitro* (279). Human cervical epithelial cells infected with *C. trachomatis* were tested similarly. We observed no difference in the percent of cell-associated or invasive gonococci in either human or murine epithelial cells in the presence or absence of *C. trachomatis* or *C. muridarum* infection, respectively (Figure 13). Interestingly, the level of gonococcal adherence to human and murine cell lines was similar despite the host restrictions associated with the human-specific pathogen *N. gonorrhoeae* (Figure 13A). However, the level of gonococcal invasion into human cells was much greater than in either of the murine cell lines (Figure 13B), underscoring the importance of the host-restricted receptors in mediating gonococcal invasion of host cells.

Figure 13. Chlamydial coinfection does not result in increased association of gonococci with cultured epithelial cells.

Shown is the percentage of cell-associated (A) or invasive (B) gonococci following inoculation of monolayers of ME180, IEC4.1, or BM1.11 cells with *N. gonorrhoeae* in the presence (white bars) or absence (gray bars) of a pre-existing chlamydial infection. ME180 human cervical epithelial cells were pre-infected with *C. trachomatis* and the IEC4.1 and BM1.11 murine epithelial cells were pre-infected with *C. muridarum.*

Figure 13. Chlamydial coinfection does not result in increased association of gonococci with cultured epithelial cells.



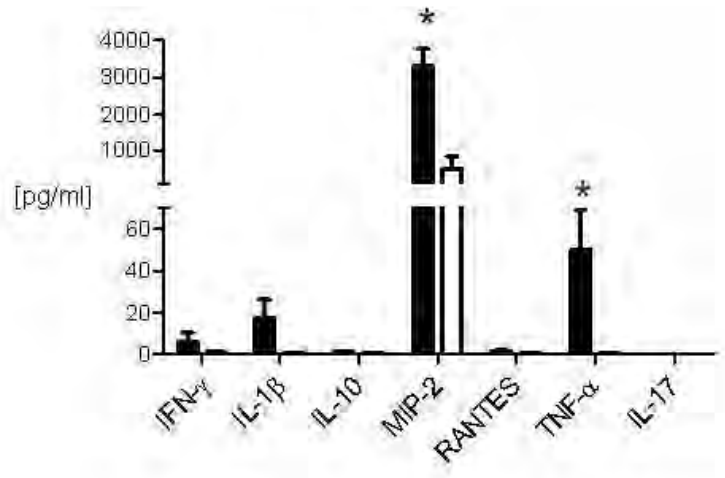
C. muridarum *infection alters the immune environment*

Because we observed a difference in gonococcal colonization as early as day one post-inoculation with *N. gonorrhoeae*, we hypothesized that immunological differences may exist as a result of chlamydial infection that could confer a more hospitable environment for *N. gonorrhoeae*. We therefore measured the concentration of seven different cytokines and chemokines in vaginal swab suspensions that are reported to play a role in both gonococcal and chlamydial infection (MIP-2, IL-1β, TNF-α, IFN-γ, RANTES, IL-10, and IL-17) in *C. muridarum* infected or mock-infected (control) mice at the time point that corresponds to inoculation with *N. gonorrhoeae*. Significant increases in the localized concentrations of the inflammatory mediators MIP-2 and TNF-α were detected in chlamydia-infected versus control mice (Figure 14).

Figure 14. Vaginal levels of inflammatory mediators MIP-2 and TNFα are increased in C. muridarum-*infected mice on day 0, prior to inoculation with* N. gonorrhoeae.

Concentrations (pg/ml) in genital tract secretions of mice infected with *C. muridarum* (solid bars) and uninfected mice (white bars) are shown from a single experiment (n = 9-10 mice per group, *$p < 0.05$).

Figure 14. Vaginal levels of inflammatory mediators MIP-2 and TNFα are increased in C. muridarum-*infected mice on day 0, prior to inoculation with* N. gonorrhoeae.

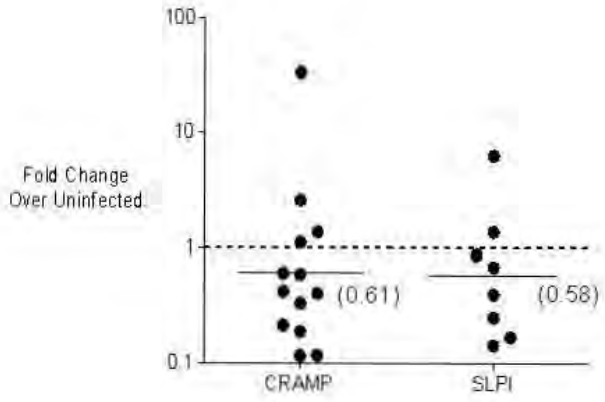


Several reports suggest that levels of MIP-2 and TNF-α can be kept in check by antimicrobial peptides, such as cathelicidin-related antimicrobial peptide (CRAMP) and secretory leukocyte peptidase inhibitor (SLPI), which also function as immunomodulatory molecules (138, 178, 208). Both CRAMP and SLPI are known to be effective against Gram-negative organisms and thus, their absence may help to explain an increase in gonococcal colonization (171). Therefore, we measured the expression of these two genes in chlamydia-infected and uninfected mice by RT-PCR. Transcripts for CRAMP and SLPI were approximately two-fold reduced in *C. muridarum*-infected mice at the time point that corresponds to inoculation with *N. gonorrhoeae* (Figure 15). These data support the hypothesis that chlamydiae-induced alterations in the immune environment of the lower genital tract make the genital tract more permissive for *N. gonorrhoeae*.

Figure 15. Antimicrobial peptide gene expression is down-regulated in mice with a pre-existing chlamydial infection prior to inoculation with N. gonorrhoeae.

CRAMP and SLPI gene expression levels were measured by RT-PCR on vaginal material collected from mice with and without a pre-existing chlamydial infection 15 minutes prior to inoculation with *N. gonorrhoeae*. Each dot represents an individual *C. muridarum*-infected mouse as compared to the average baseline value in uninfected mice at the same time point. The line is drawn at the geometric mean with the numerical value in parenthesis. The dashed line represents a fold change of one, which is the value that corresponds to no difference between mice with and without a pre-existing chlamydial infection. Values less than one indicate down-regulation of gene expression in *C. muridarum*-infected mice.

Figure 15. Antimicrobial peptide gene expression is down-regulated in mice with a pre-existing chlamydial infection prior to inoculation with N. gonorrhoeae.



Discussion

The incidence of chlamydia and gonorrhea coinfection in the young adult population in the United States is very high as illustrated by a survey of 18-26 year olds in which approximately 70% of young adults with gonorrhea were also infected with *Chlamydia trachomatis* (199). Despite these startling numbers, little is currently known about the pathogenesis or host response to coinfection. Advances are needed in this area to reduce both the costs associated with presumptive dual antibiotic treatment and the consequences of coinfection on reproductive health. Here, we describe the first small animal model of gonococcal and chlamydial coinfection for studying host-parasite interactions specific to coinfection and for developing products that are effective against both agents. While characterizing this model, we found that coinfection of female mice differs from infection with either pathogen alone in terms of gonococcal colonization load and host response to infection.

Successful coinfection of mice with *N. gonorrhoeae* and *C. muridarum* required that we first establish chlamydial infection in the absence of exogenous hormone treatment, as described (270), and then treat with 17β-estradiol to promote susceptibility to *N. gonorrhoeae*. This sequence of infection is likely to mimic a common scenario that leads to coinfection of women as suggested by mathematical modeling of two cohort studies, which showed a large number of women are asymptomatically infected with *C. trachomatis* for 18 months or more in the absence of antibiotic treatment (86). The average length of gonococcal infection is thought to be much shorter (136). Thus, it is likely that most women with coinfection at diagnosis were either asymptomatically colonized with *C. trachomatis* for a long period of time prior to infection with *N.*

gonorrhoeae or acquired both organisms simultaneously from a coinfected partner. An obvious limitation to this model is the need to treat mice with estradiol to promote susceptibility to infection with *N. gonorrhoeae*, which we showed reduces the inflammatory response to *C. muridarum*. Female mice are differentially susceptible to many different sexually transmitted pathogens depending on stage of the estrous cycle, and therefore, mouse models of sexually transmitted infections frequently utilize hormone treatment including models of *N. gonorrhoeae* (144), *C. muridarum* (15, 21), *C. trachomatis* (338), *Mycoplasma genitalium* (191), *Candida albicans* (290), and herpes simplex virus-2 (241) infections. Here we showed that several cytokines and chemokines and vaginal PMNs were decreased in *C. muridarum*-infected mice following treatment with estradiol. This model therefore does not mimic the events that occur when women with symptomatic chlamydial infection encounter *N. gonorrhoeae*. Chlamydial infection of estradiol-treated mice may more closely mimic asymptomatic chlamydial infection in women, however, which is likely often the case when a *C. trachomatis*-infected woman encounters *N. gonorrhoeae* (86). In support of this possibility, Agrawal and colleagues observed lower levels of several cytokines and chemokines in cervical washes from *C. trachomatis*-infected asymptomatic women compared to women who were symptomatic for infection (3).

Additional limitations to the model we describe here include the use of *C. muridarum* instead of *C. trachomatis* and host restrictions inherent to the use of the human-specific pathogen *N. gonorrhoeae* in a murine system. The host response and progression of infection and disease in mice infected with *C. muridarum* more closely mimics human disease, however, than does experimental infection of mice with *C.*

trachomatis (15, 63), and the *C. muridarum* mouse model is commonly used to examine the immunobiology of chlamydial genital infection. Host restrictions that prevent murine infection with *N. gonorrhoeae* from fully mimicking human infection include the absence of colonization receptors for pili and opacity (Opa) proteins (56, 156, 213, 345) and differences in soluble complement regulatory proteins that bind the gonococcal surface to downregulate complement activation (225). *N. gonorrhoeae* also cannot use murine lactoferrin or transferrin as sources of iron (57, 175), and the gonococcal immunoglobulin A1 (IgA1) protease cannot cleave mouse IgA (166). Despite these host restrictions, studying gonococcal pathogenesis in the murine model has yielded considerable insight into the host response to infection (88, 140, 236, 317) and the role of certain gonococcal virulence factors in evasion of host defenses (146, 315, 350, 367, 368). The mouse model has also allowed the demonstration of hormonal influences on selection of phase variable Opa proteins *in vivo* (56, 144, 311) as well as the effect of certain antibiotic resistance mutations on microbial fitness (351). The increasing availability of transgenic mice in several of these host-restricted factors should allow for improved study of gonococcal chlamydial coinfection *in vivo*.

An important finding of our current study was the demonstration that higher numbers of *N. gonorrhoeae* colonized the murine genital tract when *C. muridarum* was present. This result was observed whether mice were infected with *C. muridarum* for a short period (2-4 days) or a longer interval (8-10 days) prior to challenge with *N. gonorrhoeae*. We interpret this finding as evidence that the factors responsible for increased gonococcal colonization are sustained during chlamydial infection. There are several possible explanations for the observed increased gonococcal colonization in *C.*

muridarum-infected mice, including potential differences in the availability of nutrients, colonization receptors, or innate defenses. Experiments with tissue culture cells did not support the hypothesis that *C. muridarum* alters the number of gonococci that adhere to or invade epithelial cells. We also saw no difference in the number of gonococci required to infect mice with a pre-existing chlamydial infection as might be predicted if *C. muridarum* infection enhanced the capacity of gonococci to adhere to epithelial cells in the initial stages of infection (R.A. Vonck and A.E. Jerse, unpublished observation). We therefore examined the hypothesis that host responses to *C. muridarum* may alter the immune response to *N. gonorrhoeae*. In support of this hypothesis we observed increased levels of the inflammatory mediators MIP-2 and TNF-α in chlamydiae-infected mice prior to inoculation with *N. gonorrhoeae*, which occurred concurrently with reduced transcription of genes encoding the antimicrobial peptides CRAMP and SLPI. Decreased levels of antimicrobial peptides, one of the first lines of defense at the mucosal surface, could contribute to the increased gonococcal colonization that we observed during coinfection. The report that vaginal fluids from women with *C. trachomatis* had reduced levels of SLPI (76), and the demonstration that *N. gonorrhoeae* is susceptible to CRAMP and the human cathelicidin LL37 *in vitro* are consistent with this hypothesis (309, 351). This hypothesis is supported further by a growing body of evidence that IL-17 responses (88) and TLR4-mediated responses (237), both of which lead to the production of antimicrobial peptides (96, 372), are protective against *N. gonorrhoeae*. Optimization of methods to detect and measure antimicrobial peptide concentrations in genital tract secretions, which is currently underway in our laboratory, should facilitate more in-depth

studies on the consequences of altered antimicrobial peptide responses during coinfection.

MIP-2 levels correlate with localized PMN influx in both *N. gonorrhoeae* (236) and *C. muridarum* (64) infection models and a second important difference between coinfection and infection with either pathogen alone was the higher vaginal levels of MIP-2 and greater PMNs influx in coinfected mice. Interestingly, in a recent study of patients who were admitted to genitourinary medicine clinics in the United Kingdom, women that were coinfected with *C. trachomatis* and *N. gonorrhoeae* were more likely to be symptomatic than women infected with *C. trachomatis* alone (285). The mechanisms responsible for induction of greater levels of inflammation in coinfected mice and humans remain to be elucidated. The simplest explanations are that higher concentrations of pro-inflammatory pathogen-derived ligands, due to the presence of both chlamydiae and gonococci, may have a cumulative effect or that the higher gonococcal colonization load in coinfected mice may influence the degree of PMN influx. It is also possible that a unique interplay occurs between distinct pathogen-specific signaling pathways, which results in greater inflammation. At this time only the PMN response has been investigated, and the involvement of other inflammatory cell types and signaling pathways during coinfection warrant further investigation. Other potential consequences of coinfection that could be examined in this model include the possibility that *N. gonorrhoeae* infection may reactivate a latent chlamydia infection as suggested by Batteiger *et. al.* (17) in a study of recurrent chlamydial infections. The coinfection mouse model should also facilitate the development of immunomodulatory therapies, such as toll-like receptor (TLR) agonists and antagonists, which have been proposed to be used

along with antimicrobial treatment to prevent the devastating effects of sexually transmitted infections on women's reproductive health (137).

Perhaps the largest potential consequence of the unique characteristics of coinfection that we describe here is on transmission. Studies performed by Cohen and colleagues demonstrated a dose response for experimental urethral infection of male volunteers in which the percentage of infected volunteers increased with increasing dose of *N. gonorrhoeae* (54). Thus, increased gonococcal colonization due to chlamydial infection may lead to increased transmission from a coinfected female to a male partner. Additionally, the spread of chlamydiae both to the upper reproductive tract of the infected host and to an uninfected partner may be facilitated by the host PMN response, as proposed by Rank and colleagues from studies with *C. caviae* in a guinea pig genital tract infection model (276). The increased PMN influx observed in coinfected female mice might then be expected to lead to increased transmission from a coinfected female to a male partner. Three published studies have attempted to determine whether there is a difference in the rate *N. gonorrhoeae* and *C. trachomatis* transmission to an uninfected partner in the context of either single or dual infection, and no clear difference in transmission of either organism in the case of coinfection was identified (179, 181, 188). However, investigators in these studies were unable to appropriately identify index cases and several patients included as index cases were actually negative for an infection for which their partner tested positive. Thus, as concluded by Matondo and colleagues in 1995, we believe this question requires further study (188).

In summary, the female mouse model of gonococcal and chlamydial coinfection described here is easy to manipulate and can be employed to answer questions about the

pathogenesis and host response of coinfection due to the availability of mouse-specific reagents and genetically defined mouse strains. Furthermore, due to the extensive historical data on experimental murine infection with either single pathogen, this model should allow for detailed studies on differences during coinfection. Continuing studies with this model are likely to further inform the field of gonococcal immunology and increase our knowledge of polymicrobial infections in general. Additionally, this model should be a useful system for testing new antimicrobial and immunomodulatory therapies in the context of dual infection.

Acknowledgements

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Chapter 3: Chlamydial Infection Reduces TLR4 Expression on Genital Epithelial Cells and Increases Gonococcal Colonization during Murine Coinfection

At the time of this writing, this manuscript is in preparation for submission to the Proceedings of the National Academy of Science USA as: **R.A. Vonck, T. Darville, and A.E. Jerse.** Chlamydial infection reduces TLR4 expression on genital epithelial cells and increases gonococcal colonization during murine coinfection.

The work presented in this chapter is the sole work of R.A. Vonck, with the following exceptions: A.E. Jerse advised experimentation and writing of the manuscript and T. Darville provided helpful discussion.

Abstract

Neisseria gonorrhoeae and *Chlamydia trachomatis* commonly inhabit the same host, yet little is known about the pathogenesis or host response to coinfection. We recently described a female mouse model of gonococcal and chlamydial coinfection and found that gonococcal colonization was increased in chlamydiae-coinfected mice. Here we tested the hypothesis that chlamydial infection alters the immune environment of the genital tract to be more hospitable for *N. gonorrhoeae*. BALB/c mice were first infected with *C. muridarum* and then inoculated with *N. gonorrhoeae* following treatment with 17β-estradiol to promote long-term gonococcal infection. We screened for changes in gene expression during chlamydial infection using an immune-targeted RT-PCR array.

Prior to gonococcal challenge, we observed a two-fold decrease in levels of TLR4 transcript in vaginal material from *C. muridarum*-infected mice compared to uninfected mice, despite increased transcript levels for all other TLRs examined. A significant decrease in the percent of genital epithelial cells expressing TLR4 was observed by flow cytometry in *C. muridarum*-infected mice (17.5%) compared to uninfected mice (29.1%). The biological significance of TLR4 downregulation was supported by the demonstration that gonococcal colonization was not enhanced during coinfection in TLR4-deficient mice. We conclude that *C. muridarum* infection allows for increased gonococcal colonization by reducing the expression of TLR4 in the genital tract. These results are consistent with recent findings from our laboratory that TLR4 plays a protective role during gonococcal infection and suggest that reduced expression of TLR4 during chlamydial infection may promote infection with other sexually transmitted pathogens.

Introduction

Gonorrhea and chlamydia are the two most frequently reported infections in the United States (46) and coinfection with *Neisseria gonorrhoeae* and *Chlamydia trachomatis* is sufficiently common to warrant presumptive treatment for chlamydia upon diagnosis of gonorrhea (104, 366). *C. trachomatis* and *N. gonorrhoeae* are both Gram-negative, human-specific organisms, but they inhabit different niches within the same body site. *Chlamydia spp.* are obligate intracellular organisms while *N. gonorrhoeae* is facultatively intracellular, and resides predominantly in the extracellular environment. Perhaps owing in part to this difference in cellular localization, the immune responses generated against these two pathogens are very different. The host response to

chlamydial infection is relatively well-characterized and is initiated by epithelial-derived proinflammatory cytokines (279). During chlamydial infection, chlamydiae-specific CD4+ T cells are generated and traffic to the local site of infection (210) and robust serum and vaginal antibody responses develop (202). During early infection, Th17 and Th1 cells are present in approximately equal numbers, however, as infection proceeds it is the development of a strong, exclusively Th1-response that eventually results in clearance of the organism (40, 302). The importance of IFNγ for resolution of infection underscores the importance of the Th1 response to chlamydial infection (273). The host response to *N. gonorrhoeae* is not as well understood. Recent work using the murine model of gonococcal genital infection revealed the importance of a Th17-type response, which is characterized by localized release of IL-17 and recruitment of polymorphonuclear leukocytes (PMNs) (88). These observations in mice are corroborated by a recent report that humans with gonococcal infection of the rectum and pharynges have elevated levels of circulating IL-17A and IL-23, which are cytokines that aid in the differentiation of Th17 cells (97).

The type of T-helper cell response generated against a particular pathogen is largely dependent on signaling through innate immune networks early in infection. The expression of TLRs in the female genital tract is dependent upon the location within the genital tract (i.e. vagina versus uterus), hormone state, and the presence of immune cells. Therefore, the localization of a pathogen within the genital tract, the hormonal status of the individual, and the presence of immune cells can all influence TLR signaling. It is thought that chlamydiae are recognized by toll-like receptors (TLRs) -2 and -4; however, signaling through TLR2 appears to be dominant *in vivo* and is linked to the development

of immunopathology (67, 154, 230). In contrast, signaling of *N. gonorrhoeae* through TLR4 is protective in a murine model of gonococcal infection (237) and this signaling was linked to the induction of protective Th17-type responses in mouse vaginal explants (88).

Until recently, little was known about the pathogenesis or host response to coinfection due to the lack of a model system for studying interaction between these two organisms within the host. We developed a female mouse model of gonococcal and chlamydial coinfection using *Chlamydia muridarum*, the mouse species of *Chlamydia*, and *N. gonorrhoeae*. BALB/c mice were first infected with *C. muridarum* and then inoculated with *N. gonorrhoeae* following treatment with 17β-estradiol to promote long-term gonococcal infection. Viable gonococci and chlamydiae were recovered by vaginal swab for an average of 8-10 days. Chlamydial inclusion bodies were visualized in the cervix and *N. gonorrhoeae* was observed in the vaginal lumen, deep within the vaginal tissue, and in the cervix by immunohistochemical staining. Coinfected mice had a greater influx of PMNs in the genital tract compared to mice infected with either pathogen alone. An intriguing aspect of coinfection was that a higher number of gonococci were recovered from coinfected mice as early as day one post challenge with *N. gonorrhoeae* compared to mice infected with *N. gonorrhoeae* alone and this difference was consistently sustained for at least 8 days. *C. muridarum*-infected mice had increased levels of the inflammatory mediators MIP-2 and TNFα prior to gonococcal challenge compared to uninfected mice and a concurrent decrease in transcript levels for two antimicrobial peptide genes, cathelicidin related antimicrobial peptide (CRAMP) and secretory leukocyte protease inhibitor (SLPI) (347). The observed alterations in these

innate immune factors led us to hypothesize that the host response to *C. muridarum* alters the environment of the murine genital tract to be more permissive for gonococcal colonization. Here we present evidence in support of this hypothesis and show that a decrease in the percent of TLR4-expressing epithelial cells in the genital tract is likely responsible for the enhancement of gonococcal infection by coinfection with *C. muridarum*.

Results

C. muridarum *infection alters gene transcription in mice before and after challenge with* N. gonorrhoeae

To test our hypothesis that the host response to *C. muridarum* infection results in a more permissive environment for *N. gonorrhoeae*, we first screened for alterations in gene expression following chlamydial infection that may account for increased gonococcal colonization. We measured transcript levels for 84 different genes known to be involved in innate and adaptive immunity before and after challenge with *N. gonorrhoeae* in chlamydiae-infected and uninfected BALB/c mice. Chlamydial infection was established by vaginal inoculation of $3x10^5$ inclusion forming units (IFU) of *C. muridarum* on three consecutive days. On the final day of *C. muridarum* inoculation, mice were treated subcutaneously with water-soluble 17β-estradiol to promote susceptibility to *N. gonorrhoeae* two days later as per our coinfection protocol. Control mice were also treated with estradiol, but were inoculated with buffer rather than *C. muridarum* prior to gonococcal challenge. Vaginal swab samples were collected from

chlamydiae-infected and uninfected mice immediately prior to inoculation with *N. gonorrhoeae* and immediately prior to culture on day one post-gonococcal challenge to reflect host gene expression at the time of *N. gonorrhoeae*-challenge and when increased gonococcal colonization is first observed, respectively. As reported previously, higher numbers of gonococci were recovered from mice that were coinfected with *C. muridarum* than mice that were infected with *N. gonorrhoeae* alone (Figure 16). Many differences in gene expression were detected in *C. muridarum*-infected versus control mice (Table 4).

Figure 16. Mice coinfected with C. muridarum *have increased colonization with* N. gonorrhoeae.

Coinfected mice (closed circles) and mice infected with *N. gonorrhoeae* alone (open circles) were cultured for *N. gonorrhoeae* on day one post-*N. gonorrhoeae* inoculation following collection of vaginal material for gene expression analysis. The combined results from two separate experiments are shown. Each point represents a single mouse and the line represents the geometric mean ($p = 0.038$).

Figure 16. Mice coinfected with C. muridarum *have increased colonization with* N. gonorrhoeae.

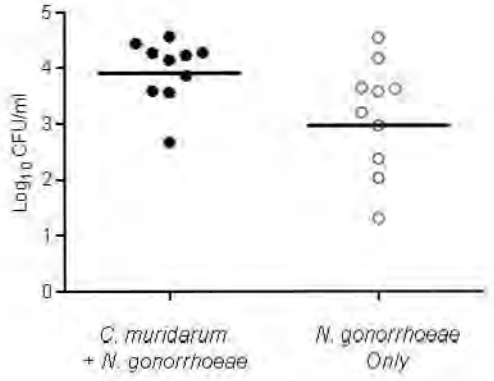


Table 4. Fold change in gene expression in chlamydiae-infected compared to uninfected mice prior to gonococcal challenge

Gene Name[a]	Symbol[b]	Mean Fold Change[c]	Range[d]
Adenosine A2a receptor	Adora2a	ND	ND
Complement component 8, alpha polypeptide	C8a	2.32	0.82-7.02
Cathelicidin antimicrobial peptide	Camp	0.49	0.22-0.91
Caspase 1	Casp1	1.41	0.44-2.75
Caspase 4, apoptosis-related cysteine peptidase	Casp4	10.17	2.18-34.50
Chemokine (C-C motif) ligand 2	Ccl2	0.68	0.09-9.26
Chemokine (C-C motif) receptor 3	Ccr3	2.65	0.85-10.82
CD14 antigen	Cd14	1.79	1.62-2.08
CD1d1 antigen	Cd1d1	0.79	0.20-6.17
CD55 antigen	Cd55	0.69	0.08-2.22
Complement factor properdin	Cfp	2.63	0.54-13.71
Conserved helix-loop-helix ubiquitous kinase	Chuk	0.39	0.09-1.84
C-type lectin domain family 7, member a	Clec7a	0.37	0.006-2.97
Collection sub-family member 12	Colec12	4.33	0.47-99.90
C-reactive protein, pentraxin-related	Crp	0.41	0.11-1.35
Chemokine (C-X-C motif) receptor 4	Cxcr4	0.69	0.12-3.82
Cytochrome b-245, beta polypeptide	Cybb	1.08	0.69-2.28
Defensin beta 4	Defb4	1.07	0.33-4.78
Deleted in malignant brain tumors 1	Dmbt1	ND	ND
Fibronectin 1	Fn1	0.22	0.02-5.64
Hemolytic complement	Hc	1.58	0.17-32.62
Heme oxygenase (decycling) 1	Hmox1	0.62	0.17-1.89
Interferon beta 1, fibroblast	Ifnb1	1.00	0.18-8.35
Interferon gamma receptor 1	Ifngr1	0.20	0.02-2.47
Interferon gamma receptor 2	Ifngr2	0.73	0.34-2.16
Inhibitor of kappaB kinase beta	Ikbkb	0.44	0.15-1.94
Interleukin 10	Il10	2.39	0.73-11.58
Interleukin 12 receptor, beta 2	Il12rb2	ND	ND
Interleukin 1 alpha	Il1a	2.25	0.37-15.56
Interleukni 1 beta	Il1b	22.34	17.96-35.88
Interleukin 1 family, member 10	Il1f10	1.26	0.62-2.52
Interleukin family, member 5 (delta)	Il1f5	0.87	0.36-1.86

Interleukin 1 family, member 6	Il1f6	0.77	0.67-0.90
Interleukin 1 family, member 8	Il1f8	0.34	0.13-0.91
Interleukin 1 family, member 9	Il1f9	0.96	0.78-1.11
Interleukin 1 receptor, type I	Il1r1	0.54	0.34-0.66
Interleukin 1 receptor, type II	Il1r2	0.56	0.37-0.91
Interleukin 1 receptor accessory protein	Il1rap	0.28	0.04-1.55
Interleukin 1 receptor accessory protein-like 2	Il1rapl2	1.76	0.64-5.01
Interleukin 1 receptor-like 2	Il1rl2	1.06	0.91-1.31
Interleukin 1 receptor antagonist	Il1rn	0.31	0.02-3.44
Interleukin 6	Il6	2.12	0.71-7.67
Interleukin-1 receptor-associated kinase 1	Irak1	0.67	0.38-1.55
Interleukin-1 receptor-associated receptor kinase 2	Irak2	1.05	0.27-2.41
Interferon regulatory factor 1	Irf1	0.90	0.68-1.26
Lactalbumin, alpha	Lalba	0.34	0.12-1.22
Lipopolysaccharide binding protein	Lbp	0.79	0.12-5.11
Lactotransferrin	Ltf	0.81	0.69-0.97
Lymphocyte antigen 96	Ly96	0.44	0.07-2.12
Lysozyme	Lyz1	9.03	1.05-4293.61
Mitogen activated protein kinase 14	Mapk14	1.07	0.04-34.51
Mitogen activated protein kinase 8	Mapk8	0.66	0.50-0.80
Macrophage migration inhibitory factor	Mif	0.71	0.45-1.21
Myeloid differentiation primary response gene 88	Myd88	0.70	0.36-1.19
Neutrophil cytosolic factor 4	Ncf4	0.61	0.20-1.26
Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	Nfkb1	1.63	0.95-2.93
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100	Nfkb2	7.20	0.41-122.38
Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	Nfkbia	1.48	0.81-2.65
NLR family, CARD domain containing 4	Nlrc4	1.53	0.16-20.62
Nitric oxide synthase 2, inducible, macrophage	Nos2	8.82	0.33-55.41
Peptidoglycan recognition protein 1	Pglyrp1	1.22	0.27-5.44
Peptidoglycan recognition protein 2	Pglyrp2	5.33	0.38-160.64
Peptidoglycan recognition protein 3	Pglyrp3	ND	ND
Pro-platelet basic protein	Ppbp	0.80	0.42-2.01
Proteoglycan 2, bone marrow	Prg2	ND	ND
Protein C	Proc	1.12	0.03-1215.43

Platelet-activating factor receptor	Ptafr	0.72	0.36-1.51
Serine (or cysteine) peptidase inhibitor, clade A, member 1a	Serpina1a	0.48	0.19-1.55
Serine (or cysteine) peptidase inhibitor, clade E, member 1	Serpine1	1.66	0.69-3.26
Surfactant associated protein D	Sftpd	0.69	0.17-2.85
Stabilin 1	Stab1	7.44	1.10-34.72
Transforming growth factor, beta 1	Tgfb1	0.61	0.31-1.01
Toll-like receptor 1	Tlr1	2.77	0.38-45.60
Toll-like receptor 2	Tlr2	15.19	4.54-74.97
Toll-like receptor 3	Tlr3	5.04	1.77-36.20
Toll-like receptor 4	Tlr4	0.68	0.38-1.72
Toll-like receptor 6	Tlr6	12.46	0.78-64.31
Toll-like receptor 8	Tlr8	2.79	0.82-10.06
Toll-like receptor 9	Tlr9	3.99	0.08-14258.41
Tumor necrosis factor	Tnf	1.67	0.44-8.43
Tumor necrosis factor receptor superfamily, member 1a	Tnfrsf1a	1.03	0.67-1.55
Toll interacting protein	Tollip	0.42	0.32-0.59
Tnf receptor-associated factor 6	Traf6	2.96	0.73-12.51
Triggering receptor expressed on myeloid cells 1	Trem1	0.97	0.19-4.15

[a] List of genes for which primers are included in the SABiosciences Immune Targeted RT-PCR Array

[b] Symbol designations for each gene

[c] The geometric mean of the fold change for 4 chlamydiae-infected mice compared to the average baseline value from 5 uninfected mice.

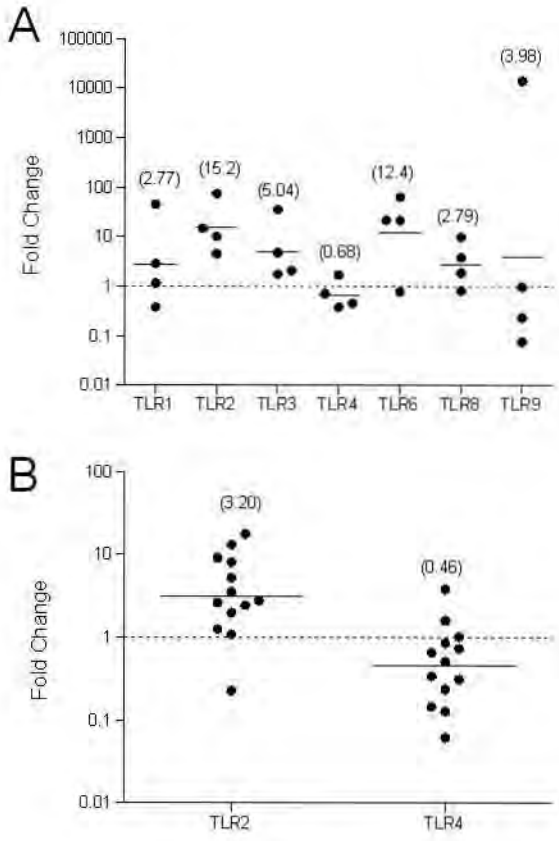
[d] Range represents the highest and the lowest fold change values of the 4 chlamydiae-infected mice.

Of particular interest was the observation that transcript levels for TLR1, -2, -3, -6, and -8 were increased in *C. muridarum*-infected mice compared to uninfected mice at the time point before gonococcal challenge, while levels of TLR4 transcript were decreased (Figure 17A). Levels of TLR9 transcript were also assessed, but were inconsistent among mice. To confirm changes in TLR expression observed by RT-PCR array, a second experiment was performed and transcript levels for TLR2 and TLR4 showed a similar increase and decrease, respectively, in *C. muridarum*-infected mice compared to uninfected mice as measured by RT-PCR (Figure 17B).

Figure 17. TLR4 transcript is decreased and TLR2 transcript is increased in C. muridarum *infected mice prior to challenge with* N. gonorrhoeae.

Gene expression levels were measured by (A) RT-PCR array and (B) individual RT-PCR reactions on vaginal material collected from mice with and without a pre-existing chlamydial infection 15 minutes prior to inoculation with *N. gonorrhoeae*. Values represent the fold change in expression in *C. muridarum*-infected mice compared to uninfected mice and each dot represents an individual mouse. The line is drawn at the geometric mean with the numerical value in parenthesis. The dashed line represents a fold change of one, where there is no difference between *C. muridarum*-infected and uninfected mice. Values less than one indicate down-regulation in *C. muridarum*-infected mice.

Figure 17. TLR4 transcript is decreased and TLR2 transcript is increased in C. muridarum *infected mice prior to challenge with* N. gonorrhoeae.



Previous work in our lab demonstrated a protective role for TLR4 during experimental gonococcal infection of female mice (237). We therefore hypothesized that reduced expression of TLR4 during chlamydial infection may alter the host response to gonococcal challenge. To test this hypothesis, we examined transcript levels for five TLR4-regulated genes (TLR2, TNFα, IL-1β, platelet activating factor receptor [Pafr], and IL-23α) in individual mice with or without a pre-existing chlamydial infection immediately prior to gonococcal challenge (day 0) and one day later. The change in transcript levels before and after gonococcal challenge was determined by comparing levels in each individual mouse on day 1 to levels on day 0. We found that in mice infected with *N. gonorrhoeae* alone, transcript levels for all five genes stayed constant or increased up to 10-fold following challenge compared to levels on day 0. However, in mice that were coinfected with *N. gonorrhoeae* and *C. muridarum* there was a 2- to 10-fold decrease in expression of these genes following gonococcal challenge compared to levels on day 0. Importantly, transcript levels in *C. muridarum*-infected mice either increased or remained unchanged following challenge with buffer instead of *N. gonorrhoeae.* Thus, the decrease in transcript levels observed following gonococcal challenge of coinfected mice was not a phenomenon caused by infection with *C. muridarum* alone (Table 5). We conclude that mice with a pre-existing chlamydial infection respond less robustly to gonococcal challenge compared to uninfected mice.

Table 5. Transcription of TLR4 regulated genes decreases in C. muridarum*-infected mice following challenge with* N. gonorrhoeae

	Mean Fold Change After *N. gonorrhoeae* Challenge[a]		
Gene of Interest	*N. gonorrhoeae* only[b]	*C. muridarum* + *N. gonorrhoeae*[c]	*C. muridarum* only[d]
TLR2	1.04	0.10	6.57
TNFα	1.36	0.37	0.71
IL-1β	3.55	0.28	1.21
Pafr	2.16	0.32	0.83
IL-23α	11.8	0.66	4.67

[a] The fold change in transcript level from day 0 to day 1 was calculated for each individual mouse and the geometric mean of these values was calculated for each group.

[b] n = 12 mice

[c] n = 9 mice

[d] n = 4 mice

TLR4 is expressed on genital epithelial cells

To better understand the potential role of TLR4 in our coinfection model we analyzed TLR4 expression on cells collected by vaginal swab by flow cytometry. Genital tract cells were collected from untreated BALB/c mice by vaginal swab and a portion of the sample was smeared onto a glass slide in order to determine stage of the estrous cycle by cytological differentiation (Figure 18A). The remaining sample was processed for flow cytometry and stained for markers of cell type and TLR4 expression. Two distinct cell populations were visible by flow cytometry (Figure 18B): namely leukocytes, which stain positive for leukocyte marker CD45.2 (Figure 18C) and epithelial cells, which stain positive for epithelial-specific cytokeratin and as a population vary considerably in size and granularity (Figure 18D). The distribution of cells in the epithelial cell and leukocyte gates correlated with distributions observed by vaginal smear (compare panel 18B to metestrus smear in 18A). TLR4 expression was only observed in the epithelial cell population when cells from mice in the metestrus stage, which contains a mix of epithelial cells and leukocytes, were examined (Figure 18E). To ensure that our staining protocol was capable of detecting TLR4 on the surface of leukocytes, we compared TLR4 expression on PMNs in the genital tract to those in whole blood. Approximately 10% of circulating PMNs expressed TLR4 (Figure 19), consistent with reports from both mice and humans (120, 205). However, the level of TLR4 expression on PMNs in the genital tract was no greater than background (Figure 19). We conclude that TLR4 is expressed on the surface of epithelial cells in the murine genital tract and differences in expression can be detected by flow cytometry.

Figure 18. Distinct populations of epithelial cells and leukocytes are visible by vaginal smear and flow cytometry on cells collected by vaginal swab.

(A) Stained vaginal smears from mice in the four different stages of the estrous cycle, as indicated, show changes in epithelial cell and leukocyte populations. A representative mouse in the metestrus stage is shown in panels B-E to illustrate the gating strategy used to identify different cell populations. (B) Scatter plot showing side scatter vs. forward scatter of cells collected from the genital tract of a representative mouse in the metestrus stage of the estrous cycle with epithelial and leukocyte populations as indicated. Histograms indicate staining for the (C) leukocyte specific marker CD45.2 and (D) epithelial specific marker cytokeratin. (E) Staining for TLR4 on the surface of cells is exclusive to genital epithelial cells. The dotted line represents the epithelial cell population and the black line represents the leukocyte population. Positive gates were drawn based on fluorescence minus one staining. Epithelial cells stain specifically for cytokeratin, while leukocytes stain for CD45.2.

Figure 18. Distinct populations of epithelial cells and leukocytes are visible by vaginal smear and flow cytometry on cells collected by vaginal swab.

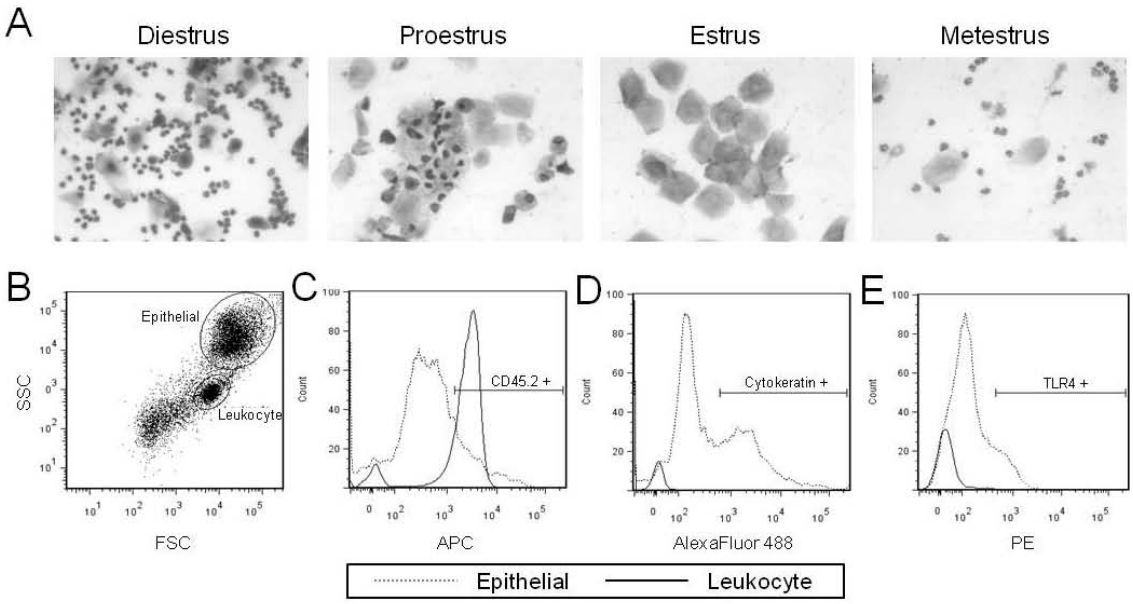
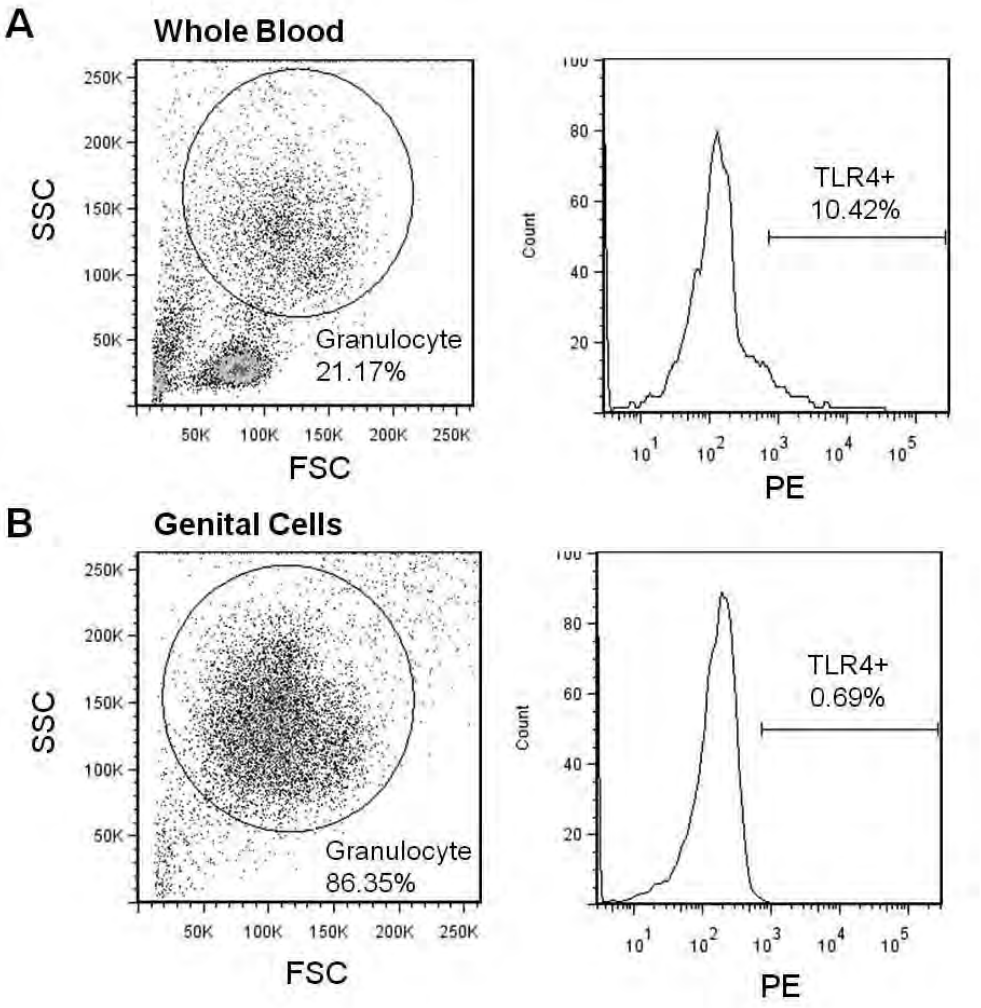


Figure 19. Circulating PMNs in the blood express TLR4, but not those found in the genital tract of normal, cycling mice.

Cells in (A) whole blood and (B) vaginal swab suspensions from mice in diestrus were stained for TLR4 and examined by flow cytometry. The granulocyte population was gated based on forward and side scatter. TLR4 expression on the surface of PMNs is shown in the panels on the right. Approximately 10% of circulating PMNs express TLR4. In contrast, less than 1% of PMNs in the genital tract expressed TLR4.

Figure 19. Circulating PMNs in the blood express TLR4, but not those found in the genital tract of normal, cycling mice.



C. muridarum infection decreases the percent of TLR4-positive epithelial cells in the genital tract

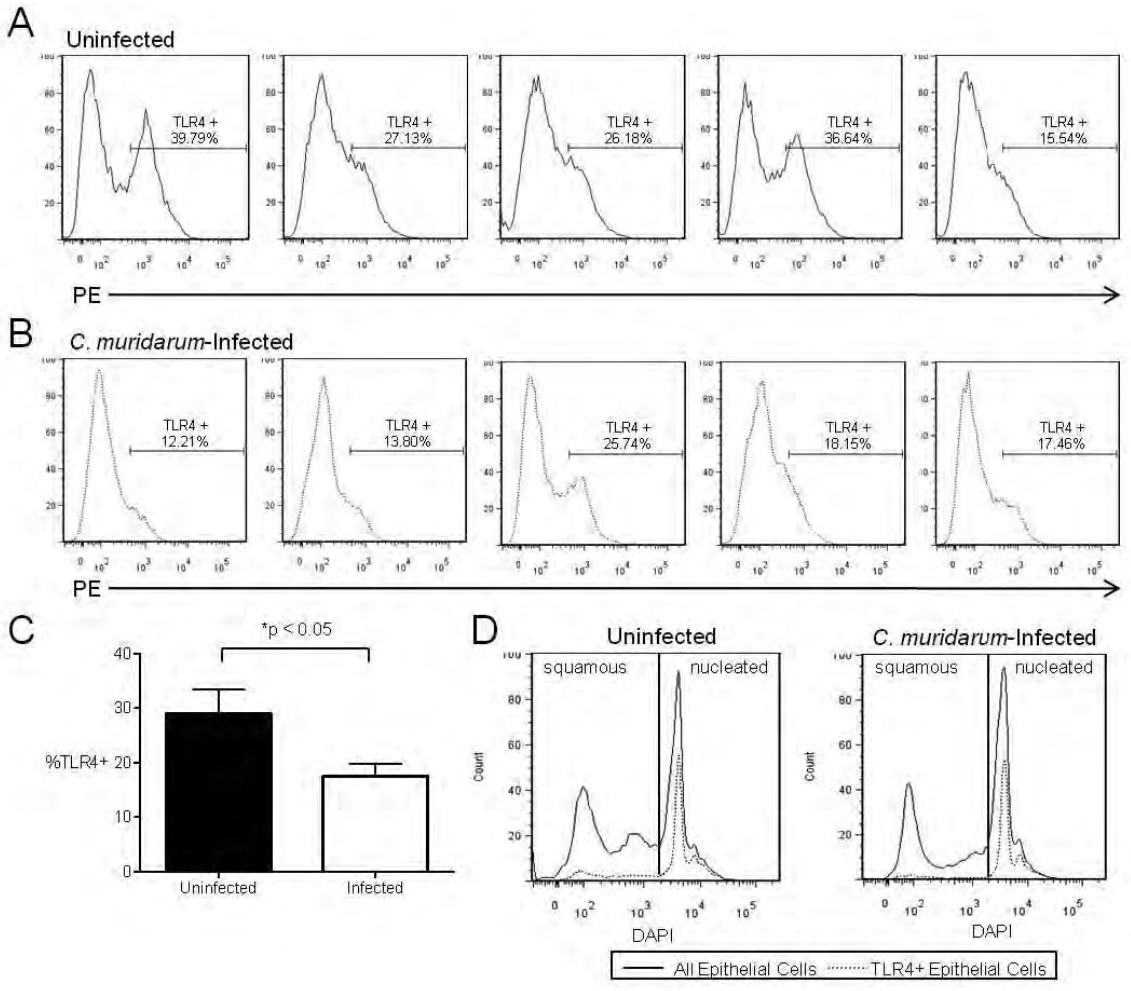
There are two possible explanations for the observed differences in gene transcription in *C. muridarum*-infected and uninfected mice. Differences in transcript levels could be the result of either changes in the type and/or number of cells present in the genital tract at the time of sample collection or changes in gene expression in individual cells. To account for these possibilities, we assessed TLR4 expression with respect to number and cell type in uninfected and *C. muridarum*-infected mice prior to inoculation with *N. gonorrhoeae*. On the final day of inoculation with *C. muridarum* or buffer, mice were treated with estradiol as per the usual protocol and vaginal swabs were collected for flow cytometry on day 0, prior to gonococcal challenge. We assessed TLR4 expression on epithelial cells in uninfected mice and mice infected with *C. muridarum*. In uninfected mice, 15.5 – 39.8% of epithelial cells stained positive for TLR4, and greater than 25% of epithelial cells were TLR4-positive in four of five mice (Figure 20A). In *C. muridarum*-infected mice, 12.2 – 25.7% of epithelial cells stained positive for TLR4, and less than 18% of epithelial cells were TLR4-positive in four of five mice (Figure 20B). Overall, the percentage of epithelial cells expressing TLR4 in *C. muridarum*-infected mice (17.5%) was significantly less than in uninfected mice (29.1%) (Figure 20C). An analysis of DNA content by DAPI staining revealed that while both squamous and nucleated epithelial cells were present in the epithelial cell population, only nucleated epithelial cells expressed TLR4 (Figure 20E). One possible explanation for the decrease in the percentage of TLR4-positive cells was that *C. muridarum* infection altered the ability of nucleated epithelial cells to differentiate into anucleated squamous epithelial

cells following estradiol treatment. However, analysis of DNA content revealed no significant difference in the percent of epithelial cells that were nucleated in *C. muridarum*-infected and uninfected mice (approximately 30-40%). Similarly, when the mean fluorescence intensity of staining for TLR4 was examined, it was unchanged in mice infected with *C. muridarum* compared to uninfected mice (Figure 20A and B). These results suggest that it is not a change in the amount of expression of TLR4 on an individual cell; rather, fewer cells express TLR4 during chlamydial infection.

Figure 20. C. muridarum *infection results in a decreased percentage of TLR4-positive nucleated epithelial cells.*

Genital cells from (A) 5 uninfected and (B) 5 *C. muridarum*-infected mice were analyzed by flow cytometry for TLR4 expression. After gating on the epithelial cell population, TLR4 expression was analyzed and is shown for individual mice in each histogram, with the percent of TLR4-positive cells indicated. (C) The mean percent of TLR4-positive epithelial cells was significantly decreased in *C. muridarum*-infected mice (white bar) compared to uninfected mice (black bar). Error bars represent SEM. (D) Analysis of DNA content by DAPI staining is shown for representative uninfected and *C. muridarum*-infected mice. The black line represents all epithelial cells and the dotted line represents TLR4-positive epithelial cells. Nucleated cells can be differentiated based on the presence of the large G1 peak with squamous cells falling to the left of this peak as they lose their nuclei during differentiation. A majority of TLR4-positive epithelial cells fall within the nucleated epithelial cell G1 peak, suggesting that nucleated epithelial cells are expressing TLR4 in the genital tract of uninfected and *C. muridarum*-infected mice.

Figure 20. C. muridarum *infection results in a decreased percentage of TLR4-positive nucleated epithelial cells.*



Coinfected TLR4-deficient mice do not exhibit increased gonococcal colonization

Based on the demonstration of a reduced percentage of TLR4-positive epithelial cells during chlamydial infection and our recent report that TLR4 is protective during gonococcal infection (237), we hypothesized that the decreased expression of TLR4 in chlamydiae-infected mice may account for the increased colonization with *N. gonorrhoeae* observed in coinfected mice. We therefore established coinfection in BALB/c mice that were deficient in the ability to signal through TLR4 (BALB/c-*LPS*^{d}) and compared gonococcal colonization to that in mice infected with *N. gonorrhoeae* alone. Wild-type BALB/c mice were tested in parallel. *C. muridarum* colonization was similar in estradiol-treated wild-type and TLR4-deficient mice (Figure 21), as reported previously for progesterone-treated mice C57BL/6 mice (67). As expected, increased gonococcal colonization was observed during coinfection with *C. muridarum* in wild-type BALB/c mice on days one and two post-inoculation with *N. gonorrhoeae* (Figure 22A). In contrast, there was no difference in the number of gonococci recovered from *C. muridarum*-infected or uninfected BALB/c-*LPS*^{d} mice (Figure 22B). These results show that the difference in TLR4 expression observed by flow cytometry is biologically significant and are consistent with the hypothesis that the absence of TLR4 signaling allows for enhancement of gonococcal colonization during chlamydial coinfection.

Figure 21. Chlamydial colonization is similar in wild-type and TLR4-deficient BALB/c mice.

Wild-type BALB/c (triangles) and TLR4-deficient BALB/c-*LPS*^{d} (squares) mice were coinfected with *C. muridarum* and *N. gonorrhoeae* and chlamydial colonization was measured on days one and two post-inoculation with *N. gonorrhoeae*. The results from 5 combined experiments are shown. Each point represents an individual mouse, the solid lines represent the geometric mean, and the dashed line represents the limit of detection.

Figure 21. Chlamydial colonization is similar in wild-type and TLR4-deficient BALB/c mice.

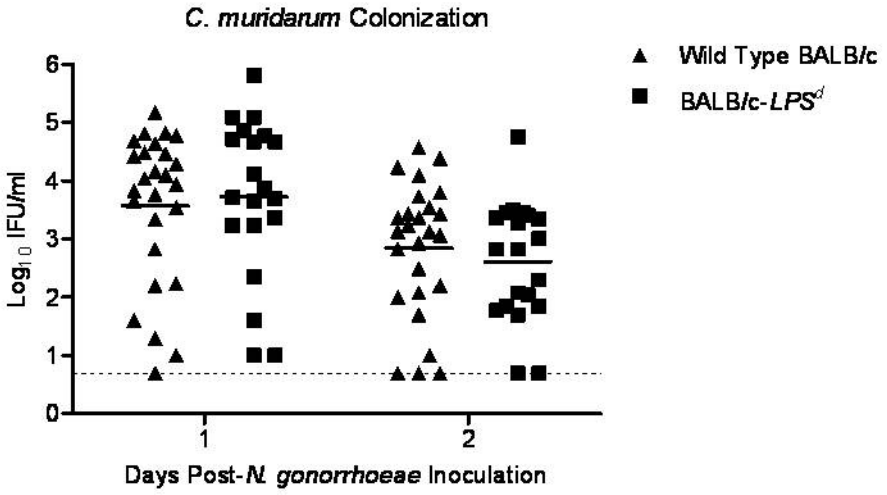
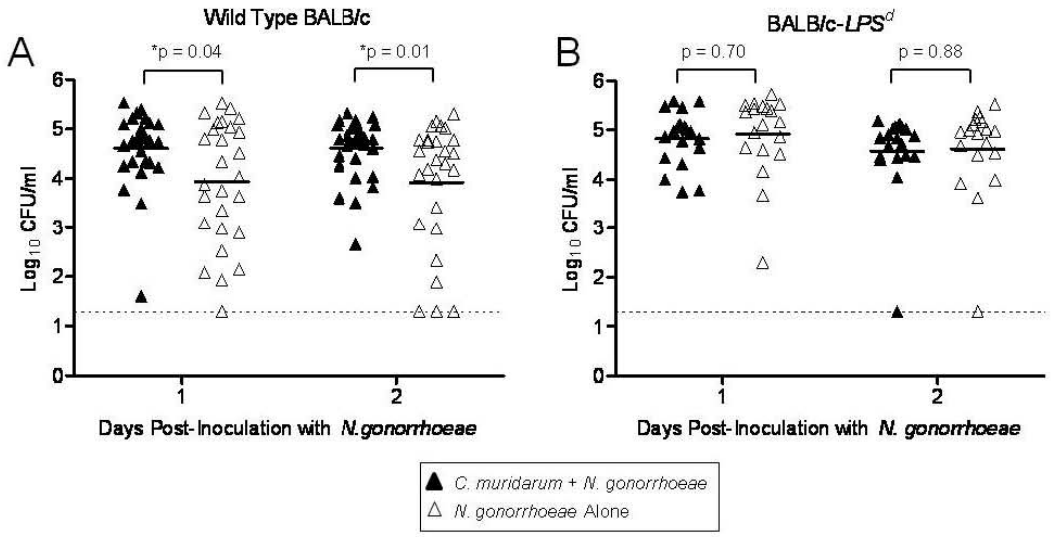


Figure 22. Gonococcal colonization is not increased in the context of coinfection with C. muridarum *in TLR4-deficient mice.*

(A) Wild-type BALB/c and (B) TLR4-deficient BALB/c-*LPS*^{d} mice were coinfected with *C. muridarum* and *N. gonorrhoeae* (solid triangles) or infected with *N. gonorrhoeae* alone (open triangles) and gonococcal colonization was measured on days one and two post inoculation with *N. gonorrhoeae*. Increased gonococcal colonization was observed in wild-type coinfected mice, but not in the TLR4-deficient mice. The results from 5 combined experiments are shown. Each point represents an individual mouse and the line represents the geometric mean (* $p < 0.05$).

Figure 22. Gonococcal colonization is not increased in the context of coinfection with C. muridarum *in TLR4-deficient mice.*



Discussion

As many as 70% of individuals with gonorrhea are coinfected with chlamydia (198), but investigation of unique aspects of the pathogenesis of coinfection have been limited by the lack of an experimental model. Mouse models of *N. gonorrhoeae* or *C. muridarum* infection have been used extensively and mimic human disease in several ways, despite the host restrictions inherent to working with human-specific pathogens (147, 204, 270). The design of the coinfection model used in this study may mimic the events that occur when a host with a pre-existing asymptomatic chlamydial infection encounters *N. gonorrhoeae* (347). Based on the high incidence of asymptomatic chlamydial infection in women, which can last as long as two years (86), this sequence of events is likely a common scenario in naturally coinfected women. Additionally, estradiol-treated chlamydiae-infected mice have decreased levels of inflammatory mediators compared to untreated chlamydiae-infected mice, as reported for women with asymptomatic infection (3). Using this model of gonococcal and chlamydial coinfection, we consistently recover more gonococci from mice coinfected with *C. muridarum* than mice infected with *N. gonorrhoeae* alone. Here we present evidence that chlamydial infection results in decreased levels of TLR4 in the genital tract and that the absence of signaling through TLR4 in the context of coinfection allowed for increased gonococcal colonization.

TLRs recognize different pathogen-associated molecular patterns (PAMPs) and the repertoire of receptors stimulated by an individual pathogen directs the host response to infection. Recent work from our lab showed that TLR4-deficient BALB/c-*LPS*^{d} mice were less able to control gonococcal infection based on increased recovery of gonococci

compared to wild-type mice, suggesting that TLR4-mediated responses are protective for genital tract infection in mice (237). In this current study, we showed that significantly fewer genital epithelial cells express TLR4 in *C. muridarum*-infected mice than in uninfected mice. We conclude that this difference is likely responsible for increased gonococcal colonization during coinfection based on the loss of the advantage in coinfected BALB/c-*LPS*^{d} mice.

Our finding that TLR4 is expressed in the lower genital tract of female mice is consistent with findings by Soboll *et al.* that transcript for TLR4 is detectable throughout the murine genital tract (314). However, reports of TLR4 expression in the lower genital tract of women are conflicting. Evidence for the presence or absence of TLR4 in human vaginal and cervical tissue may be confounded by the loss of TLR4 expression in cultured cells (124), therefore, studies discussed here are only those that used primary cells and tissues. Immunohistochemical staining was performed on lower genital tract tissues in two studies. Pivarcsi *et al.* were able to detect expression of TLR4 (256), while Fazeli *et al.* described the staining as negative, although faint staining for TLR4 at the apical surface of the vaginal epithelium was noted (87). Interestingly, while developing a protocol for the flow cytometric detection of TLR4 in the murine genital tract, we found that TLR4 was particularly sensitive to harsh fixation techniques and freezing. Antibody binding to TLR4 was optimal only when fresh, unfixed cells were stained. These technical issues may help to explain some of the differences in TLR4 expression in the lower genital tract of women, although they would not account for the inability to detect TLR4 transcript. Three published studies tested for TLR4 expression by RT-PCR. Zariffard *et al.* detected TLR4 transcript in cells collected by cervicovaginal lavage,

however, the types of cells expressing TLR4 were not characterized (377). Similarly, Herbst-Kralovitz *et al.* also detected TLR4 transcript in primary vaginal and endocervical, but not ectocervical, epithelial cells collected from healthy donors (124). In contrast, Fichorova *et al.* did not detect TLR4 transcript in primary endocervical or ectocervical epithelial cells (90).

Epithelial cells play an important role in initiating immune responses in the female genital tract (264, 361), and their importance during chlamydial infection is highlighted by the Cellular Paradigm of Chlamydial Pathogenesis, which states that the inflammatory response generated by infected epithelial cells is necessary and sufficient to account for the chronic inflammation, tissue remodeling and scarring, and development of sequelae due to chlamydial infection (323). The role of epithelial cells in the host response to gonococcal infection is not as clear. Several genital cell lines produce proinflammatory cytokines in response to gonococcal infection *in vitro* (50, 91, 116, 184, 207, 222) and similar results were demonstrated in *ex vivo* fallopian tube culture (184). The role of epithelial cells during gonococcal infection in an *in vivo* model has not been described and in the case of female models, may be host restricted in certain adherence receptors (177, 346) and complement regulatory proteins (78, 225). However, our finding that TLR4 is expressed exclusively on the surface of nucleated epithelial cells in the genital tract of female mice suggests that these cells play an important role in initiating the immune response to gonococcal infection *in vivo*.

It is currently unclear how *C. muridarum* infection results in reduced expression of TLR4 on the surface of genital epithelial cells in mice. Chlamydial infection does not

appear to alter the rate at which nucleated epithelial cells differentiate into squamous epithelial cells, but the percentage of nucleated cells expressing TLR4 is less in *C. muridarum*-infected compared to uninfected mice. Alteration of TLR4 expression during chlamydial infection appears to occur prior to translation due to the observed decrease in TLR4 transcript in *C. muridarum*-infected mice. TLR4 transcription may be altered directly in chlamydiae-infected cells or indirectly in all genital epithelial cells via the host response to chlamydial infection. Direct alteration of TLR4 transcription in chlamydiae-infected cells is an attractive hypothesis. Chlamydiae secrete several effector proteins into the host cell cytoplasm via a type three secretion system. *C. trachomatis* and *C. muridarum* also secrete an effector protein, CT621, which appears to be translocated to the nucleus of the host cell. CT621 appears to contain a DNA binding structure, leading Hobolt-Pedersen and colleagues to hypothesize that CT621 may interact with host DNA to inhibit the expression of host inflammatory genes (132), perhaps even TLR4. Alternatively, murine studies of aging and chronic inflammation have demonstrated dysregulation of TLR expression at mucosal surfaces, as in the case of increased susceptibility to pneumococcal pneumonia in aged mice (128). Inflammation generated in response to chlamydial infection could result in a similar scenario whereby levels of TLR4 are decreased in bystander epithelial cells.

Based on our findings, one may speculate that the reason TLR2-signaling is dominant during chlamydial infection (154) may be due to chlamydiae-induced downregulation of TLR4, begging the question: what would happen if TLR4 was stimulated early on during chlamydial infection? An answer to this question might allow for the determination of whether TLR4 downregulation is an active process meant to help

chlamydiae survive within the host, or rather a non-specific byproduct of the inflammatory response to chlamydial infection. A major push in the field of reproductive health is the development of immunomodulators for the prevention or treatment of the consequences of sexually transmitted infection (137). TLR4 is protective during gonococcal infection (237) and if stimulation of TLR4 were also protective during chlamydial infection, a prophylactic or therapeutic immunomodulator that stimulates TLR4 may provide some protection against both *N. gonorrhoeae* and *C. trachomatis*.

TLR4 itself is unlikely to directly affect gonococcal colonization, rather the presence or absence of signaling through TLR4 leads to downstream effects that may alter colonization. Recent work using the mouse model of gonococcal infection showed that a Th17 response is generated in response to infection and that TLR4 signaling plays a role in the development of this response (88). In mice, the initial response to chlamydial infection is characterized by approximately equal numbers of Th1 and Th17 cells, but as infection proceeds, the immune response becomes polarized toward an exclusively Th1 response (302). It therefore seems likely that the initial Th17 response is actively suppressed. Reduced expression of TLR4 and inhibition of a Th17 response may, therefore, lead to increased gonococcal colonization during chlamydial coinfection. Th17-type immune responses are characterized by the production of AMPs (372), therefore, this hypothesis is supported by our finding that transcript for the AMPs CRAMP and SLPI are decreased in *C. muridarum*-infected compared to uninfected mice prior to gonococcal challenge. It is possible that AMPs are the effectors that are modulated during chlamydial infection and that decreased levels may allow for increased gonococcal colonization.

The most alarming implications of these studies are the potential for increased transmission of *N. gonorrhoeae* and the possibility that chlamydial infection enhances other genital tract infections. A dose response is observed during experimental gonococcal infection of male volunteers (54) and it is possible that a coinfected woman with a higher gonococcal colonization load would pose a greater risk of transmission to her uninfected male partner. Consistent with this hypothesis, in a study of gonococcal and chlamydial transmission, 80% of *N. gonorrhoeae*-infected female partners of men infected with gonorrhea alone were also coinfected with *C. trachomatis* (179). The potential for increased transmission of *N. gonorrhoeae* during coinfection with *C. trachomatis* is, of course, dependent on whether or not these findings are true in naturally infected women. Unfortunately, there are currently no human data regarding gonococcal colonization load or TLR4 expression during gonococcal and chlamydial coinfection.

Finally, our data also suggest the intriguing possibility that chlamydiae-induced downregulation of TLR4 on the surface of genital epithelial cells may enhance infection with other organisms for which TLR4 plays a role in protection. Several sexually transmitted pathogens have been suggested to interact with TLR4 including *Ureaplasma urealyticum* (250), *Treponema pallidum* (293), and *Trichomonas vaginalis* (376). While a protective role for TLR4 during infection with these organisms has not been established, the potential implications of long-term chlamydial infection on the outcome of infection should not be underestimated.

Materials and Methods

Bacterial propagation

Neisseria gonorrhoeae strain FA1090 was originally isolated from a female with disseminated gonococcal infection (54). Frozen stocks of piliated, OpaB-expressing FA1090 were passaged on solid GC agar containing Kellogg's supplement I (161) and 12 μM $Fe(NO_3)_3$ and incubated at 37°C in a humidified 7% CO_2 incubator. GC agar with antibiotic selection [GC-vancomycin, colistin, nystatin, trimethoprim sulfate, and streptomycin (VCNTS)] and heart infusion agar (HIA) were used to isolate *N. gonorrhoeae* and facultatively anaerobic commensal flora, respectively, from murine vaginal mucus as described (144). *Chlamydia muridarum* strain Nigg (Dr. Darville) was propagated in L929 mouse fibroblast cells (gift of Dr. Anthony T. Maurelli, Uniformed Services University, Bethesda, MD), as described (347). L929 mouse fibroblast cells were maintained in DMEM supplemented with 10% FBS and grown to large quantities in suspension culture in RPMI supplemented with 5% FBS. Solid agar, cell culture reagents, and chemicals were purchased from Difco, Quality Biological, and Sigma, respectively, unless otherwise noted.

Murine coinfection protocol

Female mice 4-6 weeks in age were coinfected with *N. gonorrhoeae* and *C. muridarum* as described (347). Briefly, anesthetized mice were inoculated with $3x10^5$ inclusion forming units (IFU) *C. muridarum* in 20 μl 2-SP buffer on three consecutive days to establish chlamydial infection. Mice infected with *N. gonorrhoeae* alone were similarly anesthetized and mock inoculated with 20 μl of 2-SP buffer. On the final day of *C. muridarum* or buffer inoculation, mice in the diestrus stage of the estrous cycle were

identified and treated with a subcutaneous injection of 0.5 mg of water-soluble 17β-estradiol (estradiol$_{ws}$, Sigma) approximately six hours following the final inoculation with *C. muridarum* or buffer, and at 2 and 4 days later. Four hours after the second dose of estradiol$_{ws}$, mice were vaginally inoculated with $1x10^6$ colony forming units (CFU) of *N. gonorrhoeae* in 20 µl of phosphate buffered saline (PBS). All mice were given vancomycin hydrochloride and streptomycin sulfate (4 mg/ml and 2 mg/ml, respectively, per injection, twice daily, i.p.) beginning two days before *N. gonorrhoeae* inoculation and maintained for seven days. Bacterial colonization load was assessed by vaginal swab daily, as described (347). For most experiments, female BALB/c mice were purchased from the National Cancer Institute in Bethesda, MD. For experiments with TLR4-deficient mice, wild-type BALB/c and C.C3-*TLR4*$^{LPS\text{-}d}$/J mice were purchased from Jackson Laboratories in Bar Harbor, Maine. C.C3-*TLR4*$^{LPS\text{-}d}$/J mice have a mutation in the signaling domain of TLR4 which inhibits TLR4 function in the BALB/c background and are denoted as BALB/c-*LPS*^{d} throughout the text. The results from 5 combined experiments are shown. For wild-type mice, n = 29 coinfected mice and 27 mice infected with *N. gonorrhoeae* alone. For BALB/c-*LPS*^{d} mice, n = 19 each coinfected and *N. gonorrhoeae* alone infected mice.

Gene expression analysis

Vaginal material was collected from *C. muridarum*-infected and uninfected mice 15 minutes prior to inoculation with *N. gonorrhoeae* on day 0 and 15 minutes prior to culture on day 1 post-inoculation in order to evaluate host gene expression levels using a PBS-soaked Dacron swab, which was suspended directly in 500 µl RNA-Later (Ambion). Samples were stored at -80°C until use. Total RNA was extracted from the samples

using the Qiagen mini-RNeasy isolation kit per the manufacturer's instructions. Gene expression was analyzed first in a general screen using the SABioesciences RT^2 Profiler PCR Array for the Mouse Innate and Adaptive Immune Response, which contains in 96-well format primers for 84 different genes involved in innate and adaptive immunity, 5 housekeeping genes, 1 control for genomic DNA contamination, 3 reverse transcription controls, and 3 positive PCR controls. RNA was converted to cDNA using the SABioscience RT^2 First Strand Kit as instructed. Real-time PCR was performed as instructed using the SABioscience RT^2 qPCR Master Mix and an ABI 7500 sequence detector. The cycle threshold (C_T) value for each gene was determined using the Sequence Detector v.1.7a software (ABI) and data were analyzed with Microsoft Excel. For analysis, β -actin was used as the housekeeping gene. Individual arrays were performed for 5 mice with and 4 mice without a pre-existing chlamydial infection. Genes of interest were selected for further analysis in a second experiment using individual RT-PCR reactions. This second experiment included 8 mice with and 8 mice without a pre-existing chlamydial infection. At the time of challenge with *N. gonorrhoeae*, of the 8 mice with a pre-existing chlamydial infection, 4 were challenged with *N. gonorrhoeae* and 4 were mock-inoculated with PBS. Vaginal material was collected as before and RNA was converted to cDNA using the SABioscience RT^2 EZ First Strand cDNA kit as instructed. cDNA reaction mixtures (20 µl total volume) were diluted to a final volume of 100 µl with nuclease-free water. Five microliters of diluted cDNA template was then subjected to PCR amplification using an ABI 7500 sequence detector in a total reaction volume of 25 µl consisting of template, 12.5 µl SYBR green master mix (ABI), and 10 µl primer mix, which contained both forward and reverse primers at concentrations of 1 µM.

The reactions were performed according to the following parameters: 10 min at 95°C, then 40 cycles at 95°C for 15 s and at 60°C for 1 min. The cycle threshold (C_T) value was determined using the Sequence Detector v.1.7a software (ABI). Data were analyzed with Microsoft Excel using the comparative C_T method ($\Delta\Delta C_T$) for relative quantification of gene expression using β -actin as the normalizer, as described (237, 347). The expression of genes of interest in each sample was normalized to β-actin. Results of the two combined experiments are shown (arrays and individual reactions). The sequence of the oligonucleotide primers and their sources are shown in Table 6.

Flow cytometry

Vaginal cells were collected for analysis by flow cytometry using a PBS-soaked polyester swab inserted into the vagina. Material collected by vaginal swab was suspended in PBS and washed by centrifugation once prior to processing using Invitrogen Fix and Perm Cell Permeabilization Reagents for detection of intracellular antigens by flow cytometry. Cells were resuspended in 50 µl wash buffer consisting of PBS + 0.1% NaN_3 + 5% FBS. Stains for the extracellular antigens TLR4 and CD45.2 were added to appropriate tubes and cells were incubated at room temperature for 15 minutes in the dark. Samples were then fixed with the addition of 100 µl Invitrogen Fixation Medium and incubated at room temperature for 15 minutes. Cells were washed with 1 ml wash buffer followed by centrifugation at 3000 rpm for 10 minutes and supernatant was aspirated. Pellets were vortexed to fully resuspend cells and 100 µl Invitrogen Permeabilization Medium was added along with stains for intracellular cytokeratin and DNA (DAPI). Samples were incubated at room temperature in the dark for 20 minutes, followed by a final wash with 1 ml wash buffer. Following centrifugation at 3000 rpm

for 10 minutes, supernatants were aspirated and cells were resuspended in 400 µl PBS for analysis. Cells were stained with AlexaFluor 488 anti-pan cytokeratin (clone AE1/AE3, 16 µg/ml, eBioscience), APC anti-mouse CD45.2 (clone 104, 5 µg/ml, eBioscience), PE anti-mouse TLR4 (clone UT41, 10 µg/ml eBioscience), and DAPI (0.5 µg/ml, Sigma). During each analysis, compensation was performed using beads stained with each fluorophore or cells stained with DAPI alone. Positive gates for each stain were set using fluorescence minus one controls. Flow cytometry was performed in the USUHS Biomedical Instrumentation Center Flow Cytometry Core using the BD LSRII Flow Cytometer. Data were collected and results were analyzed using FACS Diva and FlowJo software. To optimize the flow cytometry protocol and to determine a gating strategy, vaginal cells were collected from normal, cycling mice that had been allowed to acclimate to the animal facility for at least 10 days. Stage of the estrous cycle was assessed by cytological differentiation of stained vaginal smears and epithelial cell and leukocyte populations were identified by staining for cytokeratin and CD45, respectively. To determine TLR4 expression in the genital tract of *C. muridarum*-infected and uninfected mice prior to gonococcal challenge, mice were infected with *C. muridarum* or mock inoculated with buffer as described above and genital cells were collected from mice on day 0. Five mice with and five mice without a pre-existing chlamydial infection were included and samples were analyzed individually. To compare TLR4 expression on PMNs in whole blood and in the genital tract, peripheral blood was collected from three mice and treated with BD Immunolyse to remove red blood cells prior to staining as described. Vaginal swabs were collected from three mice in the diestrus stage of the

estrous cycle, when the greatest number of PMNs in present, and stained as described above for comparison.

Statistical analysis

P-values reported for all experiments represent the results of unpaired, two-tailed t-tests between *C. muridarum*-infected and uninfected mice or coinfected mice and mice infected with *N. gonorrhoeae* alone. Significance is set at $p < 0.05$.

Animal use assurances

All animal experiments were conducted in the laboratory animal facility at the Uniformed Services University, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, under a protocol approved by the university's Institutional Animal Care and Use Committee.

Table 6. Oligonucleotide sequences used in this study

	Primer Sequence (5'-3')		
Gene	**Forward**	**Reverse**	**Source**
TLR2	CGTTGTTCCCTGTGTTGCT	AAAGTGGTTGTCGCCTGCT	This Study
TLR4	TTCACCTCTGCCTTCACTACA	GGGACTTCTCAACCTTCTCAA	(193)
TNFα	ACGGCATGGATCTCAAAGAC	GTGGGTGAGGAGCACGTAGT	(236)
IL-1β	CGCAGCAGCACATCAACAAGAGC	TGTCCTCATCCTGGAAGGTCCACG	(369)
Pafr	AGCAGAGTTGGGCTACCAGA	TGCGCATGCTGTAAAACTTC	(128)
IL-23α	Commercial	Commercial	SABioscience
β-actin	GCGCAAGTACTCTGTGTGGA	CATCGTACTCCTGCTTGCTG	(236)

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Chapter 4: Discussion

1.0 Preface

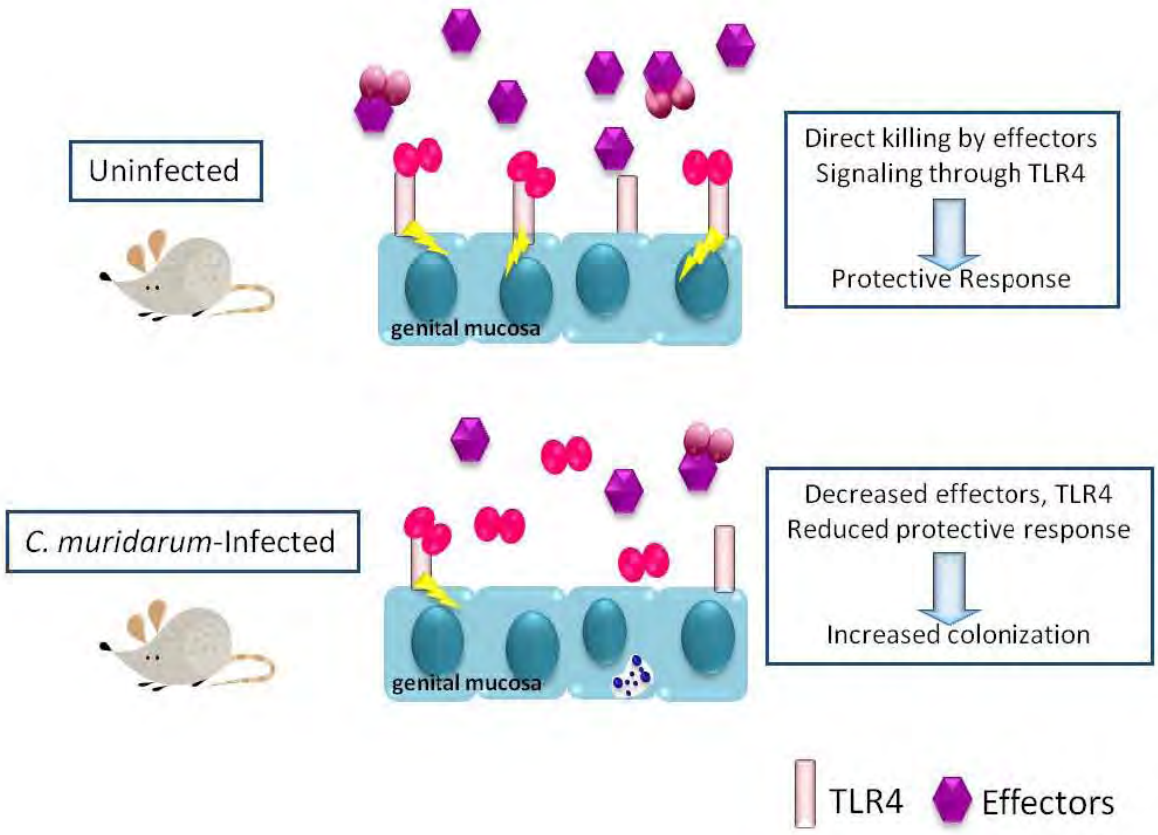
Despite the high frequency of coinfection with *N. gonorrhoeae* and *C. trachomatis*, until recently studies of the interaction between these two pathogens within the host have been hindered by the lack of an experimental model. In this work, we described the development and characterization of a female mouse model of coinfection with *N. gonorrhoeae* and *C. muridarum*, the mouse strain of *Chlamydia*. Upon characterization of the model we found that coinfection was different from infection with either pathogen alone in terms of both colonization and host response. Significantly more gonococci were recovered from the genital tract of chlamydiae-infected mice than mice infected with *N. gonorrhoeae* alone. Similarly, there was a greater PMN influx in the genital tract of coinfected mice than in mice infected with either pathogen alone. Further studies into the mechanism responsible for the difference in gonococcal colonization revealed that infection with *C. muridarum* caused decreased expression of two antimicrobial peptides (AMPs), CRAMP and SLPI, and TLR4 in the genital tract compared to uninfected mice and that the phenotype of increased colonization with *N. gonorrhoeae* during coinfection is absent in TLR4-deficient mice. Based on these findings, we propose a model for increased gonococcal colonization during chlamydial coinfection in which decreased levels of antimicrobial effectors and TLR4-expressing epithelial cells alters the environment of the genital tract such that it is more permissive for gonococcal infection (Figure 23). Correlation of these studies with findings in

naturally infected humans, the impact of this work, and potential future applications of the coinfection model are discussed below.

Figure 23. Model for increased gonococcal colonization during chlamydial coinfection

In uninfected mice (top panel), nucleated epithelial cells that express TLR4 and antimicrobial effectors are present at the surface of the genital mucosa. Upon infection with *N. gonorrhoeae* (pink circles), there is some direct killing of gonococci by the antimicrobial effectors and TLR4 is stimulated (lighting bolt), ultimately resulting in a protective response. In contrast, in *C. muridarum*-infected mice (bottom panel, chlamydiae shown as blue circles), fewer nucleated epithelial cells express TLR4 and there is a decrease in the presence of antimicrobial effectors. Upon infection with *N. gonorrhoeae*, fewer gonococci are killed immediately by antimicrobial effectors and there is less signaling through TLR4, resulting in reduced protection and increased gonococcal colonization.

Figure 23. Model for increased gonococcal colonization during chlamydial coinfection



2.0 Limitations of the Model

There are several limitations that prevent the gonococcal and chlamydial coinfection model described here from fully mimicking natural coinfection in humans. Limitations include the use of human-specific *N. gonorrhoeae* in a murine system, use of the mouse pathogen *C. muridarum* rather than the human pathogen *C. trachomatis*, treatment of mice with exogenous 17β-estradiol, and administration of antibiotics to control normal flora.

Host restrictions inherent to the use of the human-specific pathogen *N. gonorrhoeae* in a murine system that prevent murine infection with *N. gonorrhoeae* from fully mimicking human infection include the absence of colonization receptors for pili and opacity (Opa) proteins (56, 156, 213, 345) and differences in soluble complement regulatory proteins that bind the gonococcal surface to downregulate complement activation (225). *N. gonorrhoeae* also cannot use murine lactoferrin or transferrin as sources of iron (57, 175), and the gonococcal immunoglobulin A1 (IgA1) protease cannot cleave mouse IgA (166). Despite these host restrictions, studying gonococcal pathogenesis in the murine model has yielded considerable insight into the host response to infection (88, 140, 236, 317) and the role of certain gonococcal virulence factors in evasion of host defenses (146, 315, 350, 367, 368). The mouse model has also allowed the demonstration of hormonal influences on selection of phase variable Opa proteins *in vivo* (56, 144, 311) as well as the effect of certain antibiotic resistance mutations on microbial fitness (351). The increasing availability of transgenic mice in several of these host-restricted factors should allow for improved study of gonococcal and chlamydial coinfection *in vivo*.

An additional limitation of the model we describe here is the use of the mouse pathogen *C. muridarum* instead of the human pathogen *C. trachomatis*. The host response and progression of infection and disease in mice infected with *C. muridarum* more closely mimics human disease, however, than does experimental infection of mice with *C. trachomatis* (15, 63), and the *C. muridarum* mouse model is commonly used to examine the immunobiology of chlamydial genital infection. Mouse models that use human strains of *C. trachomatis* require higher inocula, have a lower peak bacterial load, and demonstrate relatively quick eradication of infection compared to infection with *C. muridarum* (252). Moreover, pathology is limited to the lower genital tract and there is a more pronounced delay of clearance in IFNγ or IFNγ-receptor knock-out mice (142, 150), likely due to a discordance in the IFNγ-response of the host with the IFNγ-defenses of the organism (190, 286). Upper genital tract pathology has only been demonstrated following direct inoculation of large numbers of *C. trachomatis* into the uterine horns or ovarian bursa (336, 337).

In our model of coinfection, mice are treated with exogenous 17β-estradiol following establishment of infection with *C. muridarum* in order to promote long term colonization with *N. gonorrhoeae*. Mouse models of sexually transmitted infections frequently utilize hormone treatment including models of *N. gonorrhoeae* (144), *C. muridarum* (15, 21), *C. trachomatis* (338), *Mycoplasma genitalium* (191), *Candida albicans* (290), and herpes simplex virus-2 (241) infections. Here we showed that several cytokines and chemokines and vaginal PMNs were decreased in *C. muridarum*-infected mice following treatment with estradiol. This model therefore does not mimic the events that occur when women with symptomatic chlamydial infection encounter *N.*

gonorrhoeae. Chlamydial infection of estradiol-treated mice may more closely mimic asymptomatic chlamydial infection in women, however, which is likely often the case when a *C. trachomatis*-infected woman encounters *N. gonorrhoeae* (86). In support of this possibility, Agrawal and colleagues observed lower levels of several cytokines and chemokines in cervical washes from *C. trachomatis*-infected asymptomatic women compared to women who were symptomatic for infection (3).

Finally, the antibiotic treatment required to prevent the overgrowth of commensal flora associated with estradiol treatment (144) may limit the ability of this model to fully represent the interaction between gonococci and chlamydiae in the genital tract of normal women with intact commensal flora. It is possible that one pathogen or the other may influence the normal flora of the genital tract, therby having an effect on the other pathogen. Under the influence of antibiotics, some commensals are still present in the genital tract of mice. Lactobacilli are frequently isolated over the course of an experiment and it is possible that other organisms are present, such as anaerobic bacteria, although the difference in the flora present in the genital tract of mice in the presence or absence of antibiotic treatment has not been evaluated. Therefore, the effect of normal flora on gonococcal and chlamydial coinfection cannot be determined using this model.

3.0 Correlation of Findings with Natural Infection

The purpose of animal modeling is to reproduce conditions in the human host. Therefore, correlation of observations made using animal models with findings from human studies is an important part of model validation. Mouse models of single pathogen infection with *N. gonorrhoeae* and *C. muridarum* as models of gonorrhea and chlamydia, respectively, have been used extensively and mimic human disease in many

ways. It is therefore likely that our model of coinfection with *N. gonorrhoeae* and *C. muridarum* will also have similarities to natural coinfection in humans. While information about coinfected individuals is limited, comparisons can be made between this model and human infection with regards to the method by which coinfection is established and the development of symptomology. Further comparisons can be made regarding modulation of AMP and TLR4 expression in the female genital tract. There are still many unknowns, however, especially about the effects of coinfection on pathogen transmission and disease severity and host and bacterial factors that may favor coinfection.

3.1 Method of Coinfection

The method by which coinfection is established in the mouse coinfection model was a major factor of concern during model development. Due to the difference in susceptibility of female mice to *N. gonorrhoeae* and *C. muridarum* during different stages of the estrous cycle, we were unable to coinfect mice with both organisms simultaneously. Following estradiol treatment mice were refractory to chlamydial infection, likely due to the relative absence of susceptible, nucleated epithelial cells in the stratified, keratinized, squamous epithelium that develops under the influence of estradiol. However, once chlamydial infection was established it was not altered by estradiol treatment, as described by others (163). Therefore, we chose to establish infection with *C. muridarum* first, and then inoculate with *N. gonorrhoeae* following treatment with estradiol to promote susceptibility to gonococcal infection. When levels of several different cytokines and chemokines were examined in *C. muridarum*-infected mice, we observed a decrease in many of the inflammatory mediators associated with

chlamydial infection in mice that were treated with estradiol compared to mice that were not. Interestingly, similar decreases in levels of inflammatory markers were observed in women who were asymptomatically infected with *C. trachomatis* compared to women with symptomatic infection (3). Taken together, these data suggest that our model may most accurately mimic acquisition of *N. gonorrhoeae* by women with a pre-existing, asymptomatic chlamydial infection. The frequency of such long-term, asymptomatic infections in women is thought to be quite high (86) and we therefore believe that our model accurately reflects a common scenario by which women become coinfected with these two organisms.

3.2 Symptomology

Mice coinfected with *N. gonorrhoeae* and *C. muridarum* have an increased PMN influx when compared to mice infected with either pathogen alone. Two human studies suggest that coinfection results in a greater level of symptomology than infection with either organism alone. A study of adolescents in high schools throughout the United States found that subjects were more likely to report symptoms (i.e. genital discharge, itching, or pain and burning upon urination) when they were coinfected with *N. gonorrhoeae* and *C. trachomatis* than when they were infected with either pathogen alone (227). Similarly, *C. trachomatis*-infected women seeking treatment at genitourinary medicine clinics in the United Kingdom were more likely to report symptoms when they were also infected with *N. gonorrhoeae* (285). Because symptomatic infection is generally defined as discharge with PMNs, coinfection in mice may accurately mimic conditions that lead to the increased probability of symptoms in humans. It is not currently known why an individual develops symptomatic versus asymptomatic infection

with either organism. In mice, different mouse strains display differences in PMN influx and cytokine response during infection with either *N. gonorrhoeae* or *C. muridarum* suggesting that host genetic factors play a role in the development of symptoms (63, 237). Utilization of the coinfection model system with different mouse strains may therefore be a valuable tool for elucidating host and bacterial factors that may play a role in the development of symptoms.

3.3 Antimicrobial Peptides

A potential explanation for the increased gonococcal colonization observed in mice coinfected with *C. muridarum* is a decrease in levels of protective AMPs present at the genital mucosa. Prior to inoculation with *N. gonorrhoeae*, mice infected with *C. muridarum* had decreased levels of transcript for the AMPs CRAMP and SLPI when compared to uninfected mice. Consistent with this finding, levels of SLPI were decreased in vaginal secretions collected from women with several different genital infections, including chlamydia (76). In contrast to this observation, stimulation with purified *E. coli* LPS or inflammatory cytokines in the lung results in increased production of SLPI, which led Draper and colleagues to hypothesize that SLPI production may be differentially regulated in the genital tract or that microbial factors can destroy SLPI (76). Additionally, further studies demonstrated that increased levels of inflammatory proteases, such as neutrophil elastase, reduced production of SLPI (327). Although the mechanism is not clear, it is possible that AMPs are the effectors that are modulated during chlamydial infection and that decreased levels may allow for increased gonococcal colonization, as shown in Figure 23. Continuation of this line of investigation may yield interesting information about the regulation of AMP expression

in the female genital tract and the AMPs that are most effective against *N. gonorrhoeae in vivo*.

3.4 TLR4 in the Female Genital Tract

The presence of TLR4 on lower genital tract epithelial cells has been a subject of debate in the field of reproductive immunology. Our finding that TLR4 is expressed on nucleated epithelial cells within vaginal swab suspensions from mice is consistent with the observation by Soboll and colleagues that transcript for TLR4 is present throughout the female murine genital tract (314). This result also correlates with the detection of TLR4 in the lower genital tract of women by RT-PCR and immunohistochemical staining (124, 256, 377), although other studies in women using the same techniques suggest that TLR4 is absent from the lower genital tract (87, 90). In one particularly interesting study, TLR4 expression was compared by RT-PCR in cervical tissue from premenopausal women undergoing hysterectomy and cells collected by cervicovaginal lavage (CVL) in healthy women. Levels of TLR4 transcript were 20 times higher in cells collected by CVL than those in cervical tissue and Zariffard and colleagues concluded that the cells that express TLR4 in the lower genital tract are those nearest or within the lumen (377). Cells collected by vaginal swab in mice likely results in a population of cells similar to those collected by CVL in women, and therefore our finding of TLR4 expression on nucleated epithelial cells in the lower genital tract of female mice is consistent with TLR4 expression in women. However, Zariffard did not identify the type of cells expressing TLR4 in women (377). Interestingly, while developing a protocol for the flow cytometric detection of TLR4 in the murine genital tract, we found that TLR4 was particularly sensitive to harsh fixation techniques and freezing. Antibody binding to

TLR4 was optimal only when fresh, unfixed genital cells were used for staining. These technical issues may help to explain some of the differences observed between different groups about the expression of TLR4 in the lower genital tract of women, although they would not account for the inability to detect TLR4 transcript. Genital cell populations can be differentiated by flow cytometry in vaginal swab samples collected from women (300) and studies on the T cell response to chlamydial infection have employed genital cell collection and analysis by flow cytometry (89). Therefore, using the flow cytometry method described here in mouse cells on human samples may be a relatively simple way to resolve some of the discrepancies in the literature about TLR4 expression in the lower genital tract of women.

4.0 Potential Implication of Findings

4.1 Transmission and Ascendant Infection

One of the most alarming implications of these studies is the potential for increased transmission of *N. gonorrhoeae*. A dose response is observed during experimental infection of male volunteers with *N. gonorrhoeae* (54) and it is possible that a coinfected woman with a higher gonococcal colonization load would pose a greater risk of transmission to her uninfected male partner. Interestingly, in a study of gonococcal and chlamydial transmission, 80% of *N. gonorrhoeae*-infected female partners of men with gonorrhea alone were also coinfected with *C. trachomatis* (179). These epidemiological data support the possibility that men are infected with *N. gonorrhoeae* more readily when their female partner is coinfected with *C. trachomatis*.

Although chlamydial colonization in the lower genital tract was not altered by coinfection with *N. gonorrhoeae*, the possibility exists that coinfection does affect chlamydial transmission or ascension to the upper reproductive tract. In a guinea pig model of infection with *C. caviae*, it was hypothesized that the PMN response to infection causes the increased detachment of *C. caviae*-infected epithelial cells from the mucosal surface, which allows the detached cells to move to new locations by fluid dynamics where the organisms are released (276, 316). This movement of detached, infected cells may aid in the ascension of chlamydiae to the upper reproductive tract or to a partner during intercourse. We were unable to detect a difference in the rate of ascension or colonization load of *C. muridarum* in the upper reproductive tract of mice coinfected with *N. gonorrhoeae* vs. mice infected with *C. muridarum* alone. However, we only checked at a single time point 10 days post-inoculation with *N. gonorrhoeae*. Similarly, ascension of *N. gonorrhoeae* was only assessed at 10 days post-inoculation and no difference was observed between coinfected mice and mice infected with *N. gonorrhoeae* alone. A more thorough time course study for the ascension of both organisms may reveal differences in the timing of ascension or differences in upper reproductive tract colonization earlier in infection. The role of PMNs in these differences would be relatively easy to assess due to the availability of antibodies for the depletion of PMNs.

4.2 The Importance of Epithelial Cells and the Role of TLR4 during Gonococcal and Chlamydial Infection

The importance of epithelial cells in initiating immune responses in the female genital tract has been described (264, 361). The inflammatory response generated by

chlamydiae infected epithelial cells is thought to be necessary and sufficient to account for the chronic inflammation, tissue remodeling and scarring, and development of sequelae due to chlamydial infection (323). However, the role of epithelial cells in the host response to gonococcal infection is not quite as clear. Several genital cell lines and fallopian tube organ cultures produce proinflammatory cytokines in response to gonococcal infection *in vitro* (50, 91, 116, 184, 207, 222). The role of epithelial cells during gonococcal infection in an *in vivo* model of female genital infection has not been described. Our laboratory recently found that TLR4 plays a protective role during experimental murine infection with *N. gonorrhoeae* (237). Our finding here that TLR4 is expressed exclusively on the surface of nucleated epithelial cells in the genital tract of female mice suggests that these cells are playing an important role in initiating the immune response to gonococcal infection *in vivo*.

Recent findings suggest that a Th17-type immune response is protective during gonococcal infection and that signaling through TLR4 is linked to the development of this response (88, 97, 237). Several studies have demonstrated that TLR4 is present and bioactive in the lower genital tract of women (124, 256, 377), suggesting that TLR4 may be important during lower genital infections in women as well as mice. Our observation that downregulation of TLR4 on genital epithelial cells has an effect on gonococcal colonization in mice suggests that stimulation of epithelial cells through TLR4 may play an important role in initiating the immune response to *N. gonorrhoeae* in the female genital tract. The ability of gonococci to stimulate an inflammatory response in cultured epithelial cells in the absence of TLR4 signaling (90), however, suggests that TLR4 is not

the only signaling pathway that is activated during the interaction of host epithelial cells with *N. gonorrhoeae*.

Theoretically, chlamydiae have ligands with the potential to signal through both TLR2 and TLR4 (154); however, TLR2 signaling appears to be dominant both *in vitro* and *in vivo* and has been linked to the development of immunopathology in mice (229, 230). Based on our findings, one may speculate that the reason TLR2-signaling is dominant *in vivo* may be due to chlamydiae-induced downregulation of TLR4. This begs the question: what would happen if TLR4 were stimulated early on during chlamydial infection? An answer to this question might allow for the determination of whether TLR4 downregulation is an active process meant to help chlamydiae survive within the host, or rather a non-specific byproduct of the inflammatory response to chlamydial infection. TLR4 is protective during gonococcal infection (237) and if stimulation of TLR4 were also protective during chlamydial infection, a prophylactic or therapeutic immunomodulator that stimulates TLR4 may provide some protection against both *N. gonorrhoeae* and *C. trachomatis*.

During gonococcal and chlamydial coinfection we observed an interesting dynamic that underscores the useful nature of coinfection models in gaining insight into the immune responses important not only during coinfection, but also during single pathogen infection. The expression kinetics and impact of TLR4 downregulation during coinfection could never have been predicted based on models of infection with either *N. gonorrhoeae* or *C. muridarum* alone. The expression of TLR4 at later time points during infection with *N. gonorrhoeae* and *C. muridarum* alone and in coinfected mice has not yet been characterized. We would hypothesize that the downregulation of TLR4 in

chlamydiae-infected mice is sustained based on our finding that gonococcal colonization was increased in mice coinfected with *C. muridarum* even when challenge with *N. gonorrhoeae* was delayed to 6-8 days post-chlamydial infection.

4.3 Potential Impact of Chlamydial Infection on Other STIs

Our data suggest that chlamydiae-induced downregulation of TLR4 on the surface of genital epithelial cells is responsible for the increased gonococcal colonization observed in coinfected mice. As an innate immune receptor, many pathogens signal through TLR4. Therefore, the possibility exists that downregulation of TLR4 during chlamydial infection may alter the response to other sexually transmitted pathogens besides *N. gonorrhoeae*. Several sexually transmitted pathogens have been suggested to interact with TLR4. For example, *Ureaplasma urealyticum*, unlike other mycoplasmas which signal through TLR2 only, interacts with both TLR2 and TLR4 via a lipophilic moiety (250), two outer membrane lipoproteins of *Treponema pallidum*, the causative agent of syphilis, have been shown to result in increased expression of TLR4 on the surface of peripheral blood monocytes and macrophages following intradermal injection in humans (293), and *Trichomonas vaginalis* activates genital cells through TLR4 despite the absence of LPS on this eukaryotic organism (376). If TLR4 downregulation occurs in humans, the role that chlamydial infection may play in altering the host response during coinfection with other sexually transmitted pathogens would further support the need for chlamydial screening in women and would perhaps support the expansion of screening to young men as well.

5.0 Other Remaining Questions

5.1 Mechanism of Reduced TLR4 Expression in Chlamydiae-Infected Mice

It is currently unclear how *C. muridarum* infection results in reduced expression of TLR4 on the surface of genital epithelial cells in mice. The alteration of TLR4 expression during chlamydial infection appears to occur prior to translation due to the observed decrease in TLR4 mRNA in *C. muridarum*-infected mice. TLR4 transcription may be altered directly in chlamydiae-infected cells or indirectly in bystander epithelial cells via the host response to chlamydial infection. Direct alteration of TLR4 transcription in chlamydiae-infected cells is an attractive hypothesis. Chlamydiae secrete several effector proteins via the type three secretion system through the inclusion membrane into the host cell cytoplasm. Interestingly, *C. trachomatis* and *C. muridarum* also secrete an effector protein, CT621, which appears to be translocated to the nucleus of the host cell. CT621 has a chlamydiae-specific domain of unknown function which contains a region that resembles a DNA binding structure, leading Hobolt-Pedersen and colleagues to hypothesize that CT621 may interact with host DNA to inhibit the expression of host inflammatory genes (132), perhaps even TLR4. An *in vitro* system in which TLR4 is expressed under normal conditions may be particularly useful in this line of investigation. Unfortunately, many of the commonly used immortalized lower genital tract cell lines, such as HeLa and ME180 cells, either do not express or express very little TLR4 (124).

5.2 Host Effectors Responsible for Alterations in Gonococcal Colonization

It is unlikely that TLR4 itself is directly responsible for the change in colonization with *N. gonorrhoeae* during coinfection with *C. muridarum*. Rather, interaction of

gonococci with TLR4 likely alters the levels of antibacterial factors, such as AMPs or complement, present in the genital tract. We found that levels of transcript for two AMPs, CRAMP and SLPI, were decreased in chlamydiae-infected mice prior to gonococcal colonization. Many more antibacterial factors are present in the genital tract, however, and extensive characterization of alterations in these factors during coinfection may, as mentioned, yield information about the regulation of antimicrobial factors in the female genital tract and what kinds of effectors challenge gonococci *in vivo*. Methods for defining the host proteome of vaginal secretions from *N. gonorrhoeae*-infected mice are currently being optimized in our laboratory.

5.3 Polarization of the Immune Response

Recent work using the mouse model of gonococcal infection showed that a Th17 response is generated in response to infection and that TLR4 signaling plays an important role in the development of this response (88). In mice, the initial response to chlamydial infection is characterized by approximately equal numbers of Th1 and Th17 cells, but as infection proceeds, IFNγ levels increase and the immune response becomes polarized toward an exclusively Th1 response (302). It therefore seems likely that as chlamydial infection proceeds, the initial Th17 response is actively suppressed. Downregulation of TLR4 and inhibition of a Th17 response may, therefore, lead to increased gonococcal colonization during chlamydial coinfection. Th17-type immune responses are characterized by the production of AMPs (372), therefore, this hypothesis is supported by our finding that transcript for the AMPs CRAMP and SLPI are decreased in *C. muridarum*-infected compared to uninfected mice prior to inoculation with *N. gonorrhoeae*. Further analysis of local cytokine production and the differentiation of T-

helper cells in draining lymph nodes will be required to fully characterize how coinfection affects polarization of the immune response.

5.4 The Role of PMNs in the Female Genital Tract in Health and Disease

Normal, cycling mice in the diestrus and metestrus stages of the estrous cycle have a large number of PMNs, but in these mice we found that TLR4 was expressed almost exclusively on the surface of nucleated epithelial cells, not leukocytes. It was surprising that even during the diestrus stage of the estrous cycle, when almost all the cells collected by vaginal swab are PMNs, we saw very few leukocytes expressing TLR4. To ensure that our staining procedure was capable of detecting TLR4 on the surface of leukocytes, we compared TLR4 expression on PMNs in the genital tract to those found in whole blood. Approximately 10% of circulating PMNs expressed TLR4. This percentage is consistent with reports from both mice and humans, which suggest that only a low level of circulating PMNs express TLR4 at any given time (120, 205). However, the level of TLR4 expression on PMNs in the genital tract was no greater than background. Perhaps PMNs that migrate to the genital tract in response to MIP-2 produced under the influence of reproductive hormones (318) are not activated and therefore do not express TLR4. However, this question warrants further investigation. Whether these findings will correlate with naturally infected women is not clear. Leukocyte migration into the uterus and vagina of humans has been examined. While there is no change in the number of PMNs in the vagina over the course of the menstrual cycle (246), there is a cyclic change in the number of PMNs present in the uterus and levels are greatest around the time of menstruation (292), consistent with high levels of PMNs during the transition from metestrus to diestrus in mice.

The finding that TLR4 is not present on the surface of leukocytes in the genital tract of healthy mice raises an interesting question about the role of PMNs in the genital tract during disease. During the diestrus stage of the estrous cycle a large number of PMNs are present in the genital tract, yet these PMNs do not appear to be capable of responding quickly to stimulation with a TLR4 ligand. It would be interesting to follow TLR4 expression in the genital tract of female mice over the course of gonococcal and chlamydial coinfection as well as during single pathogen infection for two reasons. First, an increased number of PMNs expressing TLR4 observed during infection may indicate that the mechanism by which PMNs are attracted to the site of infection is different than the mechanism by which they are attracted during normal cycling, despite our observation that PMN influx in coinfected mice correlated with increased levels of MIP-2. Second, it would be interesting to determine if chlamydial infection, which downregulates TLR4 on the surface of epithelial cells, can also alter the expression of TLR4 on PMNs that flux into the genital tract in response to infection by comparison of TLR4 expression on PMNs during infection with *N. gonorrhoeae* alone.

5.5 Effects of Chlamydial Coinfection on Gonococcal Colonization in Women

The most obvious question generated following characterization of our murine model of gonococcal and chlamydial coinfection was: are these findings true in women? With regard to the increased PMN influx observed in coinfected mice compared to mice infected with *C. muridarum* or *N gonorrhoeae* alone, two studies support the validity of this finding in humans, as discussed (227, 285). However, there are currently no human data regarding gonococcal colonization load or TLR4 expression during coinfection. Unfortunately, current diagnostic strategies in the United States generally consist of non-

culture based techniques (366) and the study of gonococcal colonization in coinfected women would require quantitative culture of *N. gonorrhoeae* from women coinfected with *N. gonorrhoeae* and *C. trachomatis* and women infected with *N. gonorrhoeae* alone. *N. gonorrhoeae* isolates are obtained for the Gonococcal Isolate Surveillance Project (GISP), which has been in place since 1989 and monitors the antibiotic susceptibility of gonococcal isolates from men in 28 cities around the US (45). While it is unfortunate that these surveillance activities do not include women, it is promising that the infrastructure is already in place at these 28 sites for culture of *N. gonorrhoeae* and it would be particularly valuable for the study of coinfection if isolates were also collected from women for quantitative culture. Additional collection of genital cells by cytobrush, similar to the methods used by the Quayle laboratory, would allow for analysis of TLR4 expression by flow cytometry (89). It is our hope that publication of our findings using the murine model will motivate researchers at clinical centers to answer these important questions.

6.0 Future Applications of the Coinfection Model

Mouse models are particularly useful for the study of infectious disease due to the availability of many mouse-specific reagents and the increased availability of knock-out and transgenic mice. It is our hope that these tools can be used to study gonococcal and chlamydial coinfection further.

6.1 Upper Reproductive Tract Pathology and Infertility

Both gonococcal and chlamydial infection can cause PID and damage to the upper reproductive tract, often resulting in infertility in women. Following *C. muridarum*

infection, mice have a decreased ability to become pregnant and produce fewer embryos when they do become pregnant (69). Some epidemiologic studies suggest that gonococcal and chlamydial coinfection may increase the risk of infertility in women (183, 357). Furthermore, at least in the case of chlamydia, much of the damage to the genital tract is immune-mediated and our finding that coinfection is characterized by a greater influx of PMNs in the lower genital tract than infection with either pathogen alone suggests the possibility of increased tissue damage during coinfection. In contrast to chlamydiae, gonococci are thought to cause tissue damage directly through the release of peptidoglycan and LOS in outer membrane blebs (365). Due to the different mechanisms by which these two organisms cause tissue damage, the possibility exists that coinfection might have an additive, or even synergistic, effect on the development of pathology. The mouse coinfection model should allow for direct comparison of the effects of infection with *C. muridarum* alone, *N. gonorrhoeae* alone, or both pathogens on the development of upper reproductive tract pathology and fertility. A better understanding of the mechanisms that result in pathology during coinfection could lead to preventative therapies that would work equally well during single or dual pathogen infection. Therapies of this type could be used in women with gonorrhea and chlamydia to prevent infertility even when coinfection status is unknown.

6.2 Experimental Transmission Model

Epidemiologic studies of gonococcal and chlamydial transmission during coinfection have been inconsistent due to difficulties in identifying index cases (179, 181, 188). A controlled model system for studying transmission may help to answer questions about how coinfection may alter pathogen transmission and to more effectively design

future studies in humans. Sexual transmission of *N. gonorrhoeae* from a male chimpanzee to two female chimpanzees was demonstrated (7) and transmission in a guinea pig model of *C. caviae* has been described (272). While chimpanzees are also susceptible to chlamydial infection, limited availability and cost preclude the use of chimpanzees for studies with *N. gonorrhoeae* and *C. trachomatis*. Similarly, while the guinea pig model of *C. caviae* infection has allowed for some important observations about chlamydial transmission, especially with respect to infectious dose, very few guinea pig-specific reagents are available and the cost of purchasing and housing guinea pigs is greater than that of mice. Additionally, attempts to infect guinea pigs with *N. gonorrhoeae* were unsuccessful (Ann Jerse and Roger Rank, personal communication). A mouse model of transmission in the context of gonococcal and chlamydial coinfection would be quite useful. Male mice have been experimentally infected with *C. muridarum* following intraurethral inoculation (239), but subsequent transmission to female mice has not been described. There have been no reports of gonococcal infection in male mice. In men, either transferrin or lactoferrin is required to establish experimental gonococcal urethritis and these sources of iron are host restricted. Therefore, the absence of human transferrin or lactoferrin in the urethra of male mice may be a limiting factor for the infection of male mice with *N. gonorrhoeae.* The development of human transferrin transgenic mice may allow establishment of urethral infection of male mice with *N. gonorrhoeae* (375), solving the problem of the lack of a transmission model for coinfection.

6.3 Product Development

Perhaps the most valuable use of this model will be in the development of new therapies for the treatment of gonococcal and chlamydial coinfection and its consequences. A major push in the field of reproductive health is the development of immunomodulators for the prevention or treatment of the consequences of infection with either *N. gonorrhoeae* or *C. trachomatis* (137). However, before immunomodulatory therapies can be used appropriately in coinfected individuals, it is important to understand how the immune response to coinfection is different from infection with either pathogen alone. The mouse model of coinfection developed here will facilitate not only the study of the immune response to coinfection, but also *in vivo* testing of such therapies. Additionally, the model can be used for testing antibiotic therapies with effect against both organisms simultaneously, which would be beneficial due to the recommendation for presumptive treatment for chlamydia upon diagnosis of gonorrhea (366). An agent for treatment of both organisms with one drug may reduce the cost of treatment and screening for both organisms.

7.0 Conclusion

We have developed the first small animal model system for the study of gonococcal and chlamydial coinfection. Due to high rates of coinfection with *N. gonorrhoeae* and *C. trachomatis* and the limited knowledge about how coinfection differs from infection with either pathogen alone there was a great need for this model. We made several interesting observations regarding colonization and host response and believe that this is only the beginning of this model's utility. We observed that

coinfection with *C. muridarum* resulted in increased gonococcal colonization in the lower genital tract and that coinfected mice had a greater PMN influx than mice infected with either pathogen alone. We discovered that the increased gonococcal colonization in chlamydiae-coinfected mice is dependent on TLR4, which has opened up a large field of study. We have evidence that infection with *C. muridarum* results in decreased expression of TLR4 on the surface of nucleated epithelial cells in the murine genital tract, but the mechanism by which this downregulation occurs is currently unclear. Similarly, it is also unclear why TLR4 is downregulated during chlamydial infection and whether this is a function meant to aid in the survival of the organism or rather a non-specific byproduct of the inflammatory response to chlamydial infection. We also have evidence to suggest that levels of two AMPs, CRAMP and SLPI, may be decreased at the genital mucosa in chlamydiae-infected mice prior to infection with *N. gonorrhoeae*. AMPs can challenge gonococci *in vitro* (263, 308, 373) and it has been hypothesized that this occurs *in vivo* as well (351). Techniques for defining the proteome of mouse vaginal secretions are currently being refined in our laboratory and comparing the vaginal proteome during infection with *N. gonorrhoeae* alone with the proteome during coinfection, when the gonococcus is better able to survive, should provide information about the cocktail of antimicrobial effectors that challenge *N. gonorrhoeae* in the murine genital tract. Finally, a Th17-type response is characterized by the production of antimicrobial peptides and an influx of PMNs (96, 372) and in the mouse model of gonococcal infection, the development of a protective Th17-response was linked to signaling through TLR4 (88). Therefore, we hypothesize that the Th1 polarization of the immune response during chlamydial infection may inhibit or delay the host's ability to mount an effective Th17-

response against gonococcal infection, allowing for increased gonococcal colonization. The large number of reagents available for mouse research in addition to the ever-improving genetic systems available such as knock-out and humanized transgenic mice ensure that the mouse will continue to be a valuable surrogate host for research in this area.

Finally, and perhaps more importantly, we have raised a number of questions that can be asked and predictions to be tested using human systems and in epidemiologic studies of naturally infected humans. It is our hope that our novel demonstration that chlamydial infection modulates the immune environment of the murine genital tract to be more permissive for gonococcal infection will will encourage others to more fully evaluate the consequences gonococcal and chlamydial coinfection in humans and the effect of chlamydial coinfection on the outcome of other STIs.

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