Animal modeling of female upper reproductive tract infections due to *Neisseria gonorrhoeae* and gonococcal/chlamydial coinfection

by

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ABSTRACT

Animal modeling of female upper reproductive tract infections due to *Neisseria gonorrhoeae* and gonococcal/chlamydial coinfection

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Pelvic inflammatory disease (PID) is a source of significant morbidity in women and is often caused by upper reproductive tract infection with the common sexually transmitted pathogens *Neisseria gonorrhoeae* and *Chlamydia trachomatis*. PID due to coinfection by these pathogens is also common. PID often goes undiagnosed due to a high proportion of asymptomatic cases and can have severe sequelae such as tubal factor infertility, ectopic pregnancy, and chronic pelvic pain. *N. gonorrhoeae* is associated with especially severe PID cases, but despite this, much remains unknown about gonococcal PID, partly due to a lack of animal models with which to study *N. gonorrhoeae* upper reproductive tract infections. Small animal models are essential for studying disease pathogenesis and immunology and for testing interventions such as vaccines, antibiotics, and microbicides. Historically *N. gonorrhoeae* infections have been difficult to study in an animal model due to host restrictions and a lack of closely related organisms for surrogate models. The recent discovery that human transferrin supplementation supports

N. gonorrhoeae upper reproductive tract infection in mice has opened the door to many lines of inquiry that were previously impossible.

The work presented in this dissertation includes characterization of the host response in the human transferrin-supplemented mouse model of *N. gonorrhoeae* upper reproductive tract infection and a comparison of two different routes of inoculation. We quantify the local cytokine and chemokine response to infection, as well as the CD4 T cell response, and investigate a previously-established method for preventing immunosuppression by depleting transforming growth factor β. Furthermore, we describe methodology for establishing upper reproductive tract coinfection with *N. gonorrhoeae* and *Chlamydia muridarum*. We use this model to further investigate whether coinfection alters the bacterial burden of either pathogen, and find that while coinfection has no apparent effect on gonococcal colonization, chlamydial colonization is significantly reduced in coinfected mice when mice are inoculated simultaneously with both pathogens. We also show that despite robust colonization with both pathogens, the host response is mild and undetectable against *C. muridarum*, which is likely due to immunosuppression from the estradiol treatment required to maintain gonococcal infection in mice. Finally, we demonstrate a potential application of the coinfection model for testing antibiotics. In summary, these data represent an important advancement in the field of animal modeling of sexually transmitted infections.

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CHAPTER 1: Introduction

GONORRHEA AND CHLAMYDIA

Neisseria gonorrhoeae and *Chlamydia trachomatis* are two vastly different organisms that occupy nearly identical niches in the human body. In men, both organisms most commonly cause urethritis; in women, they cause cervicitis with or without urethritis. Both *N. gonorrhoeae* and *C. trachomatis* may also colonize the pharynx and the rectum (23, 74, 113). Both *N. gonorrhoeae* and *C. trachomatis* can infect the mucosa of the eyes (99, 144). Ocular infections due to *N. gonorrhoeae* or the sexually transmitted serovars of *C. trachomatis* occur primarily in neonates born to women with cervical infections; some serovars of *Chlamydia* are exclusively ocular (99, 144). In women, *N. gonorrhoeae* and *C. trachomatis* genital tract infections may ascend to the upper reproductive tract (URT), causing a condition called Pelvic Inflammatory Disease (PID), defined as inflammation of the upper genital tract, consisting of the uterus, fallopian tubes, and ovaries, caused by an infection (61).

Despite similarities in disease presentation, the two organisms have different lifestyles. *N. gonorrhoeae* is primarily an extracellular pathogen, although it can invade and replicate inside epithelial cells. *C. trachomatis*, on the other hand, is a strictly intracellular pathogen that requires host factors to replicate. The following is a brief overview of the basic biology and history of the two organisms.

Neisseria gonorrhoeae: basic biology and history

N. gonorrhoeae is a Gram-negative diplococcus that is highly adapted to survival within the human host. It was first isolated and confirmed to be the causative agent of

gonorrhea in 1879 by Albert Neisser (81). *Neisseria* spp. are generally considered to be microaerophilic organisms, although anaerobic growth is possible in the presence of nitrite (95).

Gonococcal attachment to host cells can occur via the type IV pilus, opacity (Opa) proteins, lipooligosaccharide (LOS), or PorB, with the latter three molecules leading to uptake by cells with the corresponding receptor (51, 74). *N. gonorrhoeae* is primarily an extracellular organism but survives inside host epithelial cells during the process of transcytosis and inside polymorphonuclear leukocytes (PMNs) after phagocytosis (121, 208).

N. gonorrhoeae is naturally competent due to expression of the ComP protein, which is a component of the type IV pilus that binds to a 10 nucleotide DNA sequence found throughout the neisserial genome (170, 227). Due in part to this natural competence, *N. gonorrhoeae* acquires genes for antibiotic resistance with ease, which has resulted in rapid development of resistance to every antibiotic used in humans (206).

***Chlamydia trachomatis*: basic biology and history**

C. trachomatis was first isolated in 1907 by Stanislaus von Prowazek and Ludwig Halberstadter (143). Initially, *C. trachomatis* was mis-identified as a protozoan, and later thought to be a virus due to its requirement for host cells in which to replicate and its ability to pass through bacterial filters (143). This perception persisted until 1966 when James Moulder showed that *C. trachomatis* contains DNA, RNA, and ribosomes, and is thus properly classified as an obligate intracellular Gram-negative bacterium (143). Fourteen serovars of *Chlamydia* have been described to date, which are divided into three biovars according to the type of infection caused (52). The trachoma biovar, consisting of

serovars A - C, causes ocular infections, which can result in blindness if repeated infections occur. Serovars D - K make up the genital tract biovar and are sexually transmitted. The lymphogranuloma venereum biovar consists of serovars L1- L3, which causes invasive anorectal or urogenital infections (52).

The developmental cycle of *Chlamydia* spp. consists of two distinct stages. Elementary Bodies (EB) are the infectious particle, are about 0.3 microns in diameter, and have little metabolic activity. When EB encounter a susceptible cell type, they attach to the cell and are endocytosed. After endocytosis, *C. trachomatis* diverts the endosome from the pathway by which it would normally fuse with lysosomes, and instead creates its own compartment within the cell called an inclusion. At this stage, the EB asynchronously differentiate into Reticulate Bodies (RB) which are the replicative form of the organism and are about 1 micron in diameter. The RB then begin to divide, and the inclusion increases in size until it occupies nearly the entire volume of the cell (143). During this time, the type III secretion system delivers effector proteins into the host cytoplasm that allow the bacteria to modulate apoptosis, inhibit phagocytosis, and acquire nutrients from the host (207). After replication of the RB, some RB are converted to EB, which are then released from the host cell via extrusion of the inclusion or host cell lysis (143).

Epidemiology and treatment

C. trachomatis and *N. gonorrhoeae* are the most common and second most common bacterial STIs, respectively. In 2012, the worldwide prevalence of *N. gonorrhoeae* among women aged 15-49 years was approximately 0.8%, with an estimated total of 78 million new infections per year (140). The estimated prevalence of

C. trachomatis in the same population and year was 4.2%, with about 131 million new cases (140). Adolescents and young adults have higher rates of *N. gonorrhoeae* and *C. trachomatis* infections. For example, in 2008, 20% of prevalent STIs and 50% of incident STIs were in women and men between the ages of 15 and 24 (174).

Currently, dual antibiotic therapy is recommended for treatment of gonorrhea, which consists of a third-generation cephalosporin combined with either doxycycline or azithromycin. The addition of doxycycline, which is a tetracycline, or the macrolide antibiotic azithromycin serves two purposes: first, to reduce the likelihood of resistance to antibiotic treatment; and second, to treat potential chlamydial coinfections. Recent studies suggest that azithromycin is less effective than doxycycline for treating rectal infections, and therefore doxycycline is preferred in cases where rectal infections are confirmed or suspected (29, 79).

Range of disease presentations

N. gonorrhoeae and *C. trachomatis* infections are classified based on the anatomic location of the infection as well as the severity of symptoms, if present. In women, most infections begin as cervical infections, approximately 50% of which are asymptomatic (113). When symptoms are present, they usually consist of a burning or itching sensation, often accompanied by a purulent neutrophil-rich cervical discharge. In about 10% to 20% of women with gonococcal or chlamydial cervicitis, the infection ascends to the endometrium and fallopian tubes to cause endometritis and/or salpingitis (61). The resulting condition is known as PID and is discussed in detail below. In men and a small percentage of women, *N. gonorrhoeae* and *C. trachomatis* urogenital infections manifest as urethritis. Symptoms including a burning or itching sensation,

painful urination, and purulent discharge; however, urethritis can also be asymptomatic. Estimates of asymptomatic infections in men range from 20% to 59% for gonorrhea and about 30% to 76% for chlamydia (113, 230). In a small percentage of cases, the infection can spread from the urethra to the epididymis, causing epididymitis (33).

Extragenital infections also represent a significant portion of all *N. gonorrhoeae* and *C. trachomatis* infections. *N. gonorrhoeae* or *C. trachomatis* rectal infections or *N. gonorrhoeae* pharyngeal infections occur in both sexes, and both organisms can also infect the conjunctivae of the eyes. About 80% - 95% of rectal and pharyngeal infections are asymptomatic, which contributes to the lack of detection of these infections, and thus increased transmission (150, 204). Both *N. gonorrhoeae* and *C. trachomatis* genital serovars can cause ophthalmia neonatorum, which occurs in approximately 30%-50% of neonates born to mothers with gonococcal or chlamydial cervical infections and *N. gonorrhoeae* is a leading cause of infant blindness in regions of the world without adequate screening or prophylaxis (99).

N. gonorrhoeae, unlike *C. trachomatis*, can disseminate from mucosal sites to cause a systemic infection. Disseminated gonococcal infection (DGI) can develop in both males and females. Only a small percentage of infections progress to DGI, and dissemination is strain-dependent, as bacterial factors including sialylation of LOS and expression of certain variants of the outer membrane porin PorB1A contribute to serum-resistance (74, 158). Various studies have estimated the risk of dissemination to be between 0.6% and 3% for women and between 0.4% and 0.7% for men (40). DGI usually results in petechial skin lesions and/or arthritis (40).

PELVIC INFLAMMATORY DISEASE

Symptoms and diagnostic criteria

Acute PID usually manifests as lower abdominal and/or pelvic pain, which is sometimes accompanied by systemic signs of infection such as fever, chills, nausea, and vomiting. Other signs and symptoms include abnormal vaginal discharge or bleeding and pain during urination or sexual activity. Cervical or adnexal tenderness are also common and may be present even in mild cases. In severe cases of PID, tubo-ovarian abscesses can form, which may rupture; hospitalization is often required in such cases. In less severe cases, endometritis and salpingitis are the primary markers of disease (61).

Diagnosis of PID is usually clinical. Consequences of delayed treatment are severe; thus, presumption of PID and immediate treatment is recommended in cases of unexplained pelvic or lower abdominal pain combined with cervical motion, uterine, or adnexal tenderness in at-risk women (42, 61). The most specific tests for diagnosing PID include endometrial biopsy to identify histopathology consistent with endometritis, transvaginal sonography or magnetic resonance imaging to detect inflammation and fluid accumulation in the fallopian tubes, and laparoscopy to detect salpingitis or peritonitis; however, these tests are rarely used except in cases of treatment failure (61). Testing for *N. gonorrhoeae*, *C. trachomatis*, and *M. genitalium* is usually done by nucleic acid amplification test; saline microscopy is used to test for *Trichomonas vaginalis* or bacterial vaginosis, which can also be associated with PID (61).

PID is also frequently inapparent, and the lack of symptoms may contribute to a delay in seeking treatment (58). Asymptomatic PID is no less likely to cause long-term sequelae. Wiesenfeld and colleagues reported in 2002 that 26% of women with *N. gonorrhoeae* cervical infection and 27% of women with *C. trachomatis* cervical infection

showed signs of subclinical PID, defined as histological endometritis in the absence of other symptoms (217). Moreover, a later prospective cohort study from the same group demonstrated that women with subclinical PID had a 40% reduced incidence of pregnancy compared to women without subclinical PID when followed for 2.5 or 2.8 years, respectively, suggesting that unrecognized PID may be a significant contributing factor to infertility (218).

Epidemiology

Approximately 750,000 cases of acute PID are diagnosed each year in the United States (195). In the 2013-2014 National Health and Nutrition Education Survey, 4.4% of sexually experienced women between the ages of 18 and 44 reported a previous diagnosis of PID (98). Extrapolated to the US population, that would indicate that approximately 2.5 million women in the US have a history of PID (98). Although gonococcal and chlamydial infection incidence has been increasing steadily, the rate of severe PID requiring hospitalization declined 68% from 1985 through 2001; in the same time period, there was a 47% decrease in ambulatory visits for PID (195). The reason for this seemingly paradoxical decline is unknown.

The risk factors for PID are similar to risk factors for acquisition of STIs in general. Women younger than 25 years and those who became sexually active at a young age are at a greater risk for PID (61). Use of non-barrier contraception including intrauterine devices (IUD) or oral contraceptives are associated with higher risk, particularly when IUD insertion was recent (183). Other risk factors for PID include having multiple sexual partners, young age, smoking, illicit drug use, lower socioeconomic status, and not being married (124, 183). Bacterial vaginosis (BV) is a

risk factor for acquisition of both gonorrhea and chlamydia; however, longitudinal studies of women with BV have not consistently found that pre-existing BV increases the risk of acquiring PID. Black women are at greater risk for PID; between 1995 and 2001, Black women were 3.5 times as likely to be hospitalized for PID as white women (195). It is unclear how much of this disparity is due to biases and inconsistencies in diagnosis and reporting versus underlying health factors (61). One possible contributing factor to racial disparities in PID was explored by Taylor and colleagues, who found that variants of Toll-like receptors 1 and 4 that are associated with African American race are also associated with increased odds of endometritis and/or URT infection (200).

Etiology

Due to the difficulty of obtaining cultures from the endometrium and fallopian tubes, the causative organism is unknown in many cases of PID. If there is evidence of a pathogen such as *N. gonorrhoeae* or *C. trachomatis* in cervical swabs, that organism is usually presumed to also be the cause of URT infection. Moreover, even when endometrial cultures are collected, in about 25-30% of cases, no pathogen can be identified at all (122). Thus, information on the etiology of PID and how different etiologies affect symptoms or outcomes of infection is limited.

N. gonorrhoeae and *C. trachomatis* are generally thought to be the leading causes of PID. Overall, *C. trachomatis* is far more prevalent than *N. gonorrhoeae*, but some smaller populations may have locally high rates of *N. gonorrhoeae*, contributing to the wide range of case numbers attributable to the two pathogens. Another source of variability is due to differences in the site cultured. In studies on PID, many investigators use cervical swabs to identify the presumptive cause of PID; fewer obtain cultures from

the endometrium and fallopian tubes. Even in studies that include samples from multiple sites, the results do not necessarily agree with one another. Wiesenfeld and colleagues reported in 2005 that among women with acute PID, 49% had positive cervical cultures for *N. gonorrhoeae*, and 36% had positive cervical cultures for *C. trachomatis*. Endometrial samples collected from the same women were only 9% positive for *N. gonorrhoeae* and 20% positive for *C. trachomatis* (220). Among women with subclinical PID, the differences between culture sites was even more pronounced, with 21% and 36% testing positive for cervical *N. gonorrhoeae* and *C. trachomatis*, respectively, compared to 3% and 10% with positive endometrial cultures for *N. gonorrhoeae* and *C. trachomatis* (220). It is not surprising, then, that the proportion of PID cases due to *N. gonorrhoeae* has been variously reported as 4%, 7%, and 25%, while chlamydial PID has been estimated at 10%, 13%, 28% and 44% (25, 71, 75, 122).

Two *Mycoplasma* species, *M. genitalium* and *M. hominis*, have also been linked to PID. *M. genitalium* was first recognized as a cause of non-gonococcal urethritis in men; it was not implicated in PID until serological samples provided evidence of a recent previous *M. genitalium* infection in 40% of women with PID symptoms and no other identifiable pathogen (126, 219). The development of nucleic acid amplification testing (NAAT) improved the ability to test endometrial tissue for *M. genitalium*, and one important study used this technique to demonstrate the presence of *M. genitalium* in the cervix or endometrium of 16% of women with endometritis and in 2% of women without endometritis (34). Later studies detected *M. genitalium* infections in 13% to 15% of women with acute PID (66, 182). *M. hominis* has also been associated with endometritis,

although this is usually in the context of BV, and whether it is independently linked with PID is unclear (31, 135).

Interestingly, while PID due to *C. trachomatis* is generally more common, *N. gonorrhoeae*-related PID can be more severe. Two studies in 2013 and 2014 examined hospital records for women in Australia to find PID-related hospital admissions after a diagnosis of either gonorrhea or chlamydia. The investigators found that women with gonococcal PID were more likely to have severe disease than women with chlamydial PID and were over three times as likely to require hospitalization for treatment (166, 167). Similarly, the Pelvic Inflammatory Disease Evaluation and Clinical Health (PEACH) trial found that women with gonococcal PID and those with coinfections of two or more pathogens sought medical care approximately 6 days earlier than women with chlamydial or mycoplasma PID (202). The delayed time to seek treatment is likely explained by the severity of symptoms experienced, since gonococcal PID is more strongly associated with clinical features such as fever, adnexal tenderness, mucopurulent cervicitis, and elevated peripheral leukocyte counts when compared to PID caused by *C. trachomatis* or *M. genitalium* (180). However, not all studies support a greater severity for gonococcal PID. In 1994, Heinonen and Miettinen utilized laparoscopy and endometrial histopathology to differentiate between severe and mild PID and attempted to correlate these findings with cultures obtained from the endometrium and fallopian tubes (71). They found that *C. trachomatis* and *N. gonorrhoeae* were equally likely to be found in both severity groups, although *C. trachomatis* was isolated more frequently from the fallopian tubes in women with severe disease. Strikingly, in 9 out of 10 cases, anaerobic bacteria (primarily *Bacteroides* and *Prevotella* spp.) were associated with

severe disease characterized by pyosalpinx or tubo-ovarian abscess, and nearly all of those infections were also polymicrobial (71).

BV, a condition of dysbiosis in the lower reproductive tract, has also been linked to development of PID; however, because BV is not caused by one pathogen and has a wide range of symptoms, the association between the two conditions has been difficult to determine. BV may cause cervicitis even in the absence of other pathogens, and women who have BV are more than twice as likely to acquire other STIs, including *N. gonorrhoeae*, *C. trachomatis*, *T. vaginalis*, HSV-2, and HIV (93). The increased risk for other infections may be due to decreased abundance of *Lactobacillus* spp. and the absence of *Lactobacillus*-produced lactic acid, hydrogen peroxide, and bacteriocins. Alternatively, microbial products associated with BV may alter mucosal immunity and raise vaginal pH, thus increasing susceptibility to STIs and the risk of bacterial ascension (201).

There have been conflicting results from studies that attempt to link BV and PID, possibly due to the numerous different diagnostic methods for the two conditions. A meta-analysis in 2013 systematically compared the results of 19 such studies (201). Nearly all eligible studies were cross-sectional analyses that did not detect temporal relationships between BV and PID. However, to our knowledge, one of the largest studies discussed was also the only prospective cohort study on the topic that has been conducted. This study, called the GYN Infections Follow-Through (GIFT) study, followed 1179 women at high risk for PID for a median of 3 years, with vaginal swabs collected every 6 − 12 months for Gram stain and culture. The GIFT study showed that when diagnosing BV by the Nugent criteria (a semi-quantitative analysis of Gram stains

to determine the relative abundance of bacterial genera), there was no increase in the risk of developing PID or endometritis (133). However, carriage of pigmented anaerobic Gram-negative rods was associated with PID, and women with the highest growth of several BV-associated organisms (including *Gardnerella vaginalis, M. hominis,* and *Ureaplasma urealyticum*) were significantly more likely to develop PID (133). These results suggest that one of the reasons for conflicting information regarding the role of BV in PID is that PID is only associated with a subset of BV cases and is dependent on the specific organisms that are present. In support of this hypothesis, Hillier and colleagues found that some bacterial species (*M. hominis*, *Peptostreptococcus* spp., *G. vaginalis*, and *Mobiluncus* spp.) were significantly associated with histological endometritis, but BV alone was not linked to endometritis (75). As molecular identification of bacterial species decreases in cost, allowing it to be utilized more frequently, the specific contribution of different vaginal bacterial species to bacterial ascension and inflammation of the URT may become easier to discern.

Treatment

Treatment of PID is usually empiric and should cover both *N. gonorrhoeae* and *C. trachomatis* in addition to anaerobes, Gram-negative rods, and streptococci (29). The recommendation of the inclusion of antibiotics effective against anaerobes is based on the observation that anaerobic bacteria are also capable of causing tubal and epithelial destruction in organ culture models; however, no studies have demonstrated that eliminating anaerobes prevents adverse outcomes (29). Thus, the CDC currently recommends treatment with either a third-generation cephalosporin plus doxycycline to treat *N. gonorrhoeae* and *C. trachomatis* infections, respectively, or clindamycin plus

gentamicin. Tetracycline may be substituted for doxycycline, and if the patient cannot tolerate either, erythromycin may be used instead (29). However, these treatment guidelines may be insufficient to cover all microbial causes of PID. One study found that ceftriaxone plus doxycycline was ineffective at eliminating endometrial *M. genitalium* infection in 41% of women, and women testing positive for *M. genitalium* were more likely to have continued pelvic pain and endometritis following treatment (66).

Post-infection sequelae

Pelvic inflammatory disease can have severe long-term sequelae. The best-studied of these sequelae is tubal factor infertility due to scarring from inflammation in the fallopian tubes. The National Health and Nutrition Examination Survey, which took place in the United States between 2013 and 2016, found that previous PID, even when treated, was associated with a four-fold increase of infertility in women between the ages of 20 and 29, and that about 25% of all women with a history of PID reported infertility (10).

Several factors are associated with increased risk of infertility following PID, including delayed treatment, multiple episodes of PID, and nongonococcal PID, defined as PID due to *C. trachomatis*, *M. genitalium*, or other or undefined causes. In the PEACH study, Hillis and colleagues found that seeking treatment 3 or more days after the onset of symptoms more than doubled the rate of infertility, from 8.3% to 19.7% (76). Previously, using data from a long-term Swedish cohort study, Westrom showed that each episode of PID approximately doubled the risk of tubal occlusion, with 12.8% developing tubal occlusion after one episode, 35.5% after two, and 75% after 3 or more infections (215). Data from the same cohort of women showed that tubal occlusion was more common after non-gonococcal PID, and Sweet suggests that the slower onset of symptoms

associated with nongonococcal PID is responsible for a delay in seeking care, thus worsening the impact on future fertility (196).

Rates of ectopic pregnancy following PID vary widely among published studies, probably due to differences in diagnostic criteria and length of follow-up. In the Swedish cohort study described above, 9% of women with laparascopically-confirmed salpingitis experienced an ectopic pregnancy (215). More recently, the PEACH study found that just 0.6% of women with PID experienced an ectopic pregnancy within 3 years of follow-up (137). A retrospective cohort study from Taiwan found that women with a previous diagnosis of PID had a cumulative incidence rate of 0.05% for ectopic pregnancy (82).

Approximately 18% - 36% of women experience chronic pelvic or abdominal pain after PID, and PID may be the cause of chronic pelvic pain of unknown origin in others (65, 137, 215). Like tubal occlusion, chronic pain was significantly increased after 2 or more episodes of PID compared to a single episode and was also increased when treatment was delayed by more than 3 days after symptom onset (65).

Hormonal effects on PID

The effects of estrogen and progesterone on reproductive tract infections are a long-standing question in the field. There are two factors that can affect the levels of ovarian hormones, and thus two different angles from which to approach the question. The first factor is the natural cycling of hormones that occurs throughout the menstrual cycle (described in detail below in “Hormonal cycles” and depicted in Fig. 1). The second is the effect of hormonal contraceptives, including the oral contraceptive pill (containing either estradiol and progesterone, or progesterone alone), as well as injectable progesterone, progesterone absorbed through the skin via an adhesive patch, and

intrauterine devices that release progesterone (69, 184). Estrogen replacement therapy in post-menopausal women is another factor, but to our knowledge no studies have examined the effect of estrogen therapy on sexually transmitted infections.

Multiple studies have examined the effect of the menstrual cycle on the appearance of PID symptoms, as well as acquisition of *N. gonorrhoeae* or *C. trachomatis* infections and ascension to the URT. McLaughlin and colleagues reported that menstruation at the time of sexual exposure to *N. gonorrhoeae* was significantly associated with acquiring *N. gonorrhoeae* cervical infection (119). Additionally, women with *C. trachomatis*, *N. gonorrhoeae*, or BV who were in the proliferative phase of their menstrual cycles (the estrogen-dominant phase that occurs between menses and ovulation) had increased likelihood of endometritis compared with women in other stages of the menstrual cycle (96). The authors suggest that this finding implies that the proliferative phase is a risk factor for ascending infection for *N. gonorrhoeae* and *C. trachomatis* as well as BV-associated organisms. Similarly, Curran reported that women with gonococcal PID are more likely to experience and report pain within the first 10 days of the menstrual cycle, during which estradiol levels are higher than progesterone compared to women with non-gonococcal PID for whom no such association was found (41). This association may be related to the later finding that women in the first five days of their menstrual cycles were slightly but significantly more likely to have positive *N. gonorrhoeae* cultures than women in any other stage of the cycle (116). Furthermore, some strains of *N. gonorrhoeae* can utilize hemoglobin as an iron source. Anderson and colleagues demonstrated that clinical isolates capable of using hemoglobin were more likely to be isolated early in the menstrual cycle and therefore hypothesized that

expression of the phase variable hemoglobin receptor may provide a selective advantage during menses (9).

Taken together, these findings suggest that the female reproductive tract may be more hospitable to *N. gonorrhoeae* during menses and the early proliferative phase of the menstrual cycle and that increased bacterial proliferation may allow increased ascension to the URT. Several possible mechanisms for this effect have been proposed. Keith suggested that menstruation may be associated with ascending infection/PID because of the dilation of the cervix while bleeding (94). Another possible explanation concerns the direction of uterine contractions throughout the cycle. During menses, normal uterine contractions are antegrade, meaning uterine content (including menstrual blood) is pushed from the fundus to the cervical end of the uterus (24). Retrograde menstrual bleeding occurs when the uterine contractions transport in the opposite direction, which is thought to be a factor in or a consequence of endometriosis; this has also been proposed as a mechanism for development of URT infections (24, 85). In contrast, during the late follicular phase near ovulation, normal uterine contractions are retrograde, presumably to increase the transport of sperm from the cervical area to the distal end of the fallopian tubes in order to facilitate fertilization (24). In support of the hypothesis that retrograde transport plays a role in URT infections, others have found a strong correlation between endometriosis and chronic endometritis, but whether there is a causal link is unclear (198).

A link between oral contraceptives and PID has long been suspected. Initially, oral contraceptives were thought to have a protective effect, and it was suggested that this was due to the thickening of cervical mucus, which provides a physical barrier to microbe

ascension, or because the reduction in menstrual blood flow makes the environment less hospitable for bacteria (94, 149). Several studies sought to investigate this connection. One such study found that among women with *C. trachomatis* infection, those with symptomatic PID were significantly less likely than those with asymptomatic PID to use oral contraceptives; no such association was found for women with *N. gonorrhoeae,* suggesting that the apparent protective effect of oral contraceptives may be due to a reduction in symptoms rather than actual protection (228). A similar study examined the duration of hormonal contraceptive use in relation to a diagnosis of chlamydial PID and the presence of anti-chlamydial antibodies; this study confirmed that the rate of chlamydial PID was lower in women who currently or previously used hormonal contraceptives. The authors also noted a negative association between the duration of hormonal contraception and anti-chlamydial IgA and IgG (189). At first, the results of these studies were taken to suggest that oral contraceptives may decrease susceptibility to chlamydial infection and protect against chlamydial PID. However, contrary to expectations, a study in monkeys using the subcutaneous salpingeal pocket model of PID, in which salpingeal tissue is implanted in the abdomen, showed that oral contraceptives did not affect chlamydial recovery or oviduct histopathology (153). Additionally, Ness and colleagues found that women with unrecognized endometritis were 4.3 times more likely to use oral contraceptives than women with recognized endometritis, suggesting that oral contraceptives may not protect against URT infection but may rather suppress symptoms so that the condition remains undiagnosed (134). In support of this hypothesis, a second study of a cohort of women with symptomatic PID found no significant

associations between use of oral contraceptives or medroxyprogesterone and URT infection with either *N. gonorrhoeae* or *C. trachomatis* (136).

Interpreting these studies is complicated, due to many different hormonal formulations of contraceptives, which have also changed over time, and any potential behavioral effects that may confound the data. In summary, oral contraceptives decrease symptoms of PID, regardless of the etiology, and thus the condition may go undiagnosed. Additionally, menses and the early proliferative phase of the menstrual cycle may increase the risk of acquiring *N. gonorrhoeae* infection and of ascension to the URT, and/or symptoms of PID. More studies are needed to clarify the mechanism of such effects and to further distinguish which are pathogen-specific.

ANIMAL MODELS

Chlamydial infection models

Animal models of URT infection and PID have primarily focused on chlamydial URT infection. Some of these models utilize *C. trachomatis*, and others use surrogate chlamydial species isolated from infections of other animals, including Chlamydia caviae and Chlamydia muridarum, isolated from guinea pigs and mice, r. A brief summary of the non-human primate and small animal models follows.

Mice, macaques, and specific-pathogen-free pigs have all been successfully infected with *C. trachomatis*; these infection models have the advantage of using the human pathogen rather than a surrogate chlamydial species. Mice are the most frequently used of these three animal species due to the convenience and cost of working with small animals. However, because *C. trachomatis* is not a natural pathogen of mice, in order to establish *C. trachomatis* genital infection, mice must be treated with progesterone, or *C.*

trachomatis must be inoculated directly into the upper reproductive tract (28, 48). One challenge of using mice to study *C. trachomatis* or *C. muridarum* infections is that there are significant differences in IFN-γ-related immunity between mice and humans, and IFN-γ is an important component of the immune response to chlamydial species. In humans, IFN-γ induces indoleamine 2,3-dioxgenase, which catabolizes tryptophan, and leads to depletion of available tryptophan for *C. trachomatis*. In contrast, in mice IFN-γ-related immunity appears to be primarily the result of induced expression of nitric oxide synthase and GTPases (4, 48, 169).

Pig-tailed macaques, unlike mice, can be infected with *C. trachomatis* without using hormone treatment and the anatomy and physiology of the macaque reproductive tract is much more comparable to humans than that of mice. Cervical infection with *C. trachomatis* can be established in macaques with bacterial recovery from the cervix for up to 15 weeks post-inoculation, with intermittent shedding (151). Repeated cervical inoculations do cause salpingitis and peritubal adhesions, and acute salpingitis can be established by intratubal inoculation (125). Several macaque studies have also utilized a subcutaneous pocket model, in which small pieces of oviduct or endometrial tissue are transplanted into subcutaneous pockets made by incisions on the abdominal wall (48, 152). The subcutaneous pocket model has the advantage of being able to test multiple different conditions on the same animal, and appears to elicit a similar immune response and histopathology as seen in the *in situ* infection model; however, any effects on fertility and long-term consequences of infection in the reproductive tract cannot be studied in this model (48). In summary, although the macaque model is ideal for many reasons,

ethical and practical considerations limit the number of non-human primates that can be used, which limits the usefulness of the model for large-scale studies.

One of the most common surrogate models utilizes *Chlamydia muridarum* (previously known as the mouse pneumonitis [MoPn] strain of *C. trachomatis*), which was originally isolated from a mouse lung, and causes ascending genital tract infection in female mice when inoculated vaginally. Advantages to this model include a highly reproducible disease course that is similar to that of *C. trachomatis* in women, although with a higher rate of ascension and URT pathology (132). Ascension of *C. muridarum* to the murine URT occurs within 5 to 8 weeks where it produces hydrosalpinx as a result of inflammation, scarring, and fibrosis (177), which prevents fluid drainage from the oviducts and reduces fertility (128). Hydrosalpinx roughly parallels tubal occlusion in women and is a useful quantifiable marker of pathogenicity and as an endpoint in determining the protection induced by various candidate vaccines and therapeutic agents (132).

Infection of guinea pigs with *Chlamydophila caviae* (formerly *Chlamydia psittaci* or *Chlamydia caviae*; also known as the agent of guinea pig conjunctivitis) is the other commonly-used small animal model for chlamydial infections. In this model, vaginal inoculation results in ascended infection with recoverable *C. caviae* in the endometrium and oviducts of about 78% of infected female guinea pigs. Approximately 20-40% of infected animals develop fibrosis and/or hydrosalpinx (160). Histopathology of these infections also closely resembles that seen in humans, with an influx of neutrophils, monocytes, and plasma cells (160). A unique feature of this model is that both male and

female guinea pigs can be infected, and sexual transmission of the infection also occurs (130).

Gonococcal infection models

Previous models of *N. gonorrhoeae* infection have lagged behind those for chlamydial infections, partly due to gonococcal host restrictions (discussed in more detail below). The majority of data on gonococcal PID is from tissue and organ culture models, and from animal studies utilizing female mice.

Unlike PID due to *C. trachomatis*, *N. gonorrhoeae*-induced PID has not been replicated in non-human primates, although a series of studies carried out in the 1970s utilized chimpanzees to test inoculation of *N. gonorrhoeae* by various routes. Urethritis was established in multiple male chimpanzees and this infection model was used to test varying infective doses of several different strains of *N. gonorrhoeae*, to determine whether immunity to infection was produced by challenging with the same or different strains, and to test a formalin-inactivated vaccine (13). Female chimpanzees were also inoculated, but this did not result in cervicitis. However, on two different occasions, sexual transmission from male chimpanzees with urethritis to female cagemates was observed. The female chimpanzees did not exhibit any signs of infection, and spontaneously cleared infection after approximately 30 days. Whether ascended infection occurred during this time was not investigated (21, 97). To date, chimpanzees are the only species other than humans in which urethritis has been established, and in which sexual transmission has been observed. Changes in ethical guidelines surrounding the use

of non-human primates have prevented further studies of this type, and to our knowledge, gonococcal PID was never established or observed in female chimpanzees.

The estradiol-treated mouse model of infection is well-established for studying infections of the lower reproductive tract (LRT). In this model, female mice are treated with estradiol prior to inoculation to promote an estrus-like state, which increases susceptibility to *N. gonorrhoeae* (92). Antibiotics are also used to suppress the overgrowth of vaginal commensal organisms that occurs with estradiol treatment. This animal model has been used extensively to study *N. gonorrhoeae* pathogenesis, to describe the immune response to infection, and to test candidate vaccines, antibiotics, and antimicrobials. However, one limitation of the model is that ascending infection to the URT only occurs in about 15-20% of mice, and recovery of gonococci from the endometrium is transient, not lasting more than 3 days post-inoculation. Due to these limitations, the estradiol-treated mouse model has not been useful for examining the pathogenesis or host response to URT infection.

In 2016, Islam and colleagues published a study describing a model in which mice were transcervically inoculated with *N. gonorrhoeae* (85). This model has subsequently been used to characterize the immune response to inoculation with high numbers of *N. gonorrhoeae* in mice in different hormonal states, both naturally cycling and treated with exogenous hormones. The authors report that the location of *N. gonorrhoeae* within uterine tissue was dependent upon hormonal state, with tissue invasion only occurring during in diestrus and progesterone-treated mice. Infection was not established in mice in either stage, based on the recovery of gonococci in less than 30% of mice by 48 hours post-inoculation. Interestingly, however, these investigators detected a pronounced local

inflammatory response in diestrus-stage mice, consisting of a dramatic increase in neutrophils and pro-inflammatory cytokines; this finding was in contrast to the response in estrus-stage mice, which was subdued and no longer apparent 18 hours after inoculation. Similarly, mice inoculated during diestrus had higher levels of *N. gonorrhoeae*-specific IgG after infection. The primary limitation of this model is that it does not produce sustained infection, which limits the use of the model to examining short-term consequences of introducing gonococci into the uterus.

The inability to establish sustained gonococcal infection in the murine URT is likely due to more severe host restriction in this body site. One adaptation of *N. gonorrhoeae* to humans is the ability to use human transferrin (hTf), an iron-binding blood plasma glycoprotein, as an iron source (138) through the expression of the two-component outer membrane receptor TbpAB. The neisserial TbpAB receptor is highly specific for hTf, and its ability to bind other mammalian transferrins is strongly correlated to the phylogenetic distance to humans; the closely related *Neisseria meningitidis* transferrin receptor is able to bind chimpanzee but not macaque transferrin (37, 62, 102, 138). Expression of either the hTf receptor or the receptor that binds human lactoferrin is required for urethral infection of male subjects (8, 38), but neither receptor is required for cervico-vaginal infection of female mice, likely due to the complementary utilization of siderophores from commensal bacteria in the LRT or the greater availability of ferrous iron in the lower pH of this body site (91). These data, combined with the observation that supplementation with hTf and hTf transgenic mice have been used to model infections caused by the closely related species *Neisseria meningitidis* (80, 145, 231), led to the hypothesis by our laboratory that hTf may be important for sustaining URT

infections in mice. In support of this hypothesis, we have shown that mice supplemented with hTf support a robust *N. gonorrhoeae* URT infection, with high numbers of live organisms recoverable from the endometrium and oviducts for up to 7-10 days post-inoculation (155). This model was originally established using transcervical inoculation to introduce bacteria into the URT, but importantly, vaginal inoculation of hTf-supplemented mice also results in ascension to the URT in approximately 60-70% of infected animals within 5 days of inoculation. Thus, the hTf-supplementation model can be used to study host and bacterial factors relating to the process of ascension as well as the consequences of URT infection.

Hormonal cycles

A key challenge in creating useful animal models for infections of the female reproductive tract is the fact that the humans have a reproductive cycle of approximately 28 days, while that of most small animals are usually shorter than the human cycle, ranging from 4 − 6 days to about 2 − 3 weeks. These cycles are referred to as the menstrual cycle and estrous cycle, respectively. The fundamental difference between the human menstrual cycle and the estrous cycle of other mammals is that the endometrium is shed during the menstrual cycle while it is reabsorbed in the estrous cycle. Other than humans, menstrual cycles are found in some primates, bats, the elephant shrew, and the spiny mouse; most other mammals have estrous cycles (17).

Menstrual cycles can be described in terms of the ovarian cycle or the uterine cycle, each of which is divided into phases based on physiological and hormonal changes (Fig. 1). The ovarian cycle consists of the follicular phase (from the beginning of menses to ovulation; days 1 to ~14), ovulation, and the luteal phase (from ovulation until the start

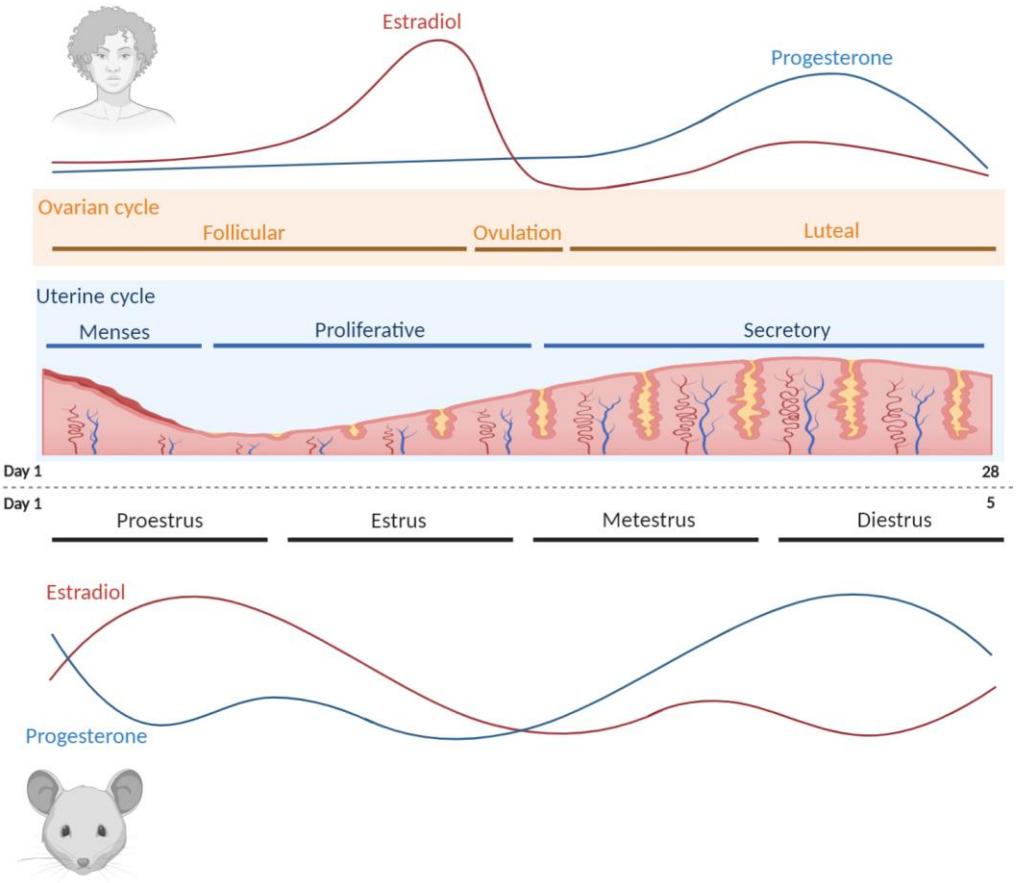

Figure 1. Menstrual and estrous cycles of humans and mice

Shown are the levels of the ovarian hormones estradiol (red) and progesterone (blue) and their approximate relative levels throughout the menstrual cycles of humans (top) and estrous cycles in mice (bottom). Also shown are the stages of the ovarian cycle and uterine cycle in humans along with the changes in uterine architecture that characterize each stage. The length of estrous cycle stages in mice are not shown to scale. Information used to create figure from (5, 17, 69, 120); figure made using Biorender.com

of the next menstrual cycle; approximately day 14 – 28) (69). The follicular phase is characterized by higher levels of estrogen and the development of follicles in the ovary. Ovulation takes place when one or two follicles release an oocyte into the oviduct, at which point progesterone levels begin to rise and estrogen levels decline (Fig. 1). Progesterone is the dominant ovarian hormone throughout the luteal phase. The uterine cycle consists of menses, the proliferative phase, and the secretory phase. The endometrium is shed during menses; during the proliferative phase, a new layer of endometrium is formed. During the secretory phase, which corresponds to the luteal phase of the ovarian cycle, the corpus luteum (formed from the remains of the ovarian follicle) produces progesterone, which increases uterine secretions and makes the endometrium more receptive for implantation (69).

Estrous cycles consist of five phases in the absence of pregnancy: proestrus, estrus, metestrus, diestrus, and anestrus, which refers to the period of quiescence that occurs in the absence of normal cycling. Proestrus corresponds to the follicular stage in humans, with high levels of estradiol; estrus corresponds to ovulation; metestrus and diestrus approximately correlate to the early and late secretory/luteal phase, with high levels of progesterone (5). The length of the estrous cycle varies widely between different species, ranging from about 4 days in mice, rats, and hamsters, to about 14-17 weeks in elephants (20, 125). Guinea pigs have longer estrous cycles than other small animals, lasting about 15-17 days (48).

The influence of ovarian hormones on the reproductive tract is complex and not yet fully understood, particularly in relation to the immune system and protection from or susceptibility to pathogens. Mice are most susceptible to chlamydial species in

progesterone-dominant phases of the estrous cycle (or when treated with exogenous progesterone); the reverse is true for guinea pigs, which are most susceptible to *C. caviae* during the estrogen-dominant phases. Rank and colleagues reported in 1982 that guinea pigs treated with estradiol prior to inoculation with *C. caviae* had significantly higher bacterial loads than untreated animals, and infection lasted about 9 days longer (162). Estradiol-treated guinea pigs also had ascending infection with inflammation in the endometrium and oviducts and developed fluid-filled oviduct cysts by 6 weeks after inoculation (162). A similar finding was reported by Barron and colleagues in 1988, after testing the effect of estrogen-dominant oral contraceptives on the course of guinea pig infection (14). However, in 1993, Rank *et al.* reported that there were no significant increases in URT pathology in guinea pigs infected during the stage of the estrous cycle when estradiol levels are naturally high (161). The highest levels of pathology were seen in animals that were infected about 6 days prior to peak serum estradiol, suggesting that the influence of estradiol on pathology is most important at the time when bacterial ascension would naturally occur (161). In contrast to guinea pigs, mice are not susceptible to vaginal inoculation of *C. muridarum* when treated with estradiol, and progesterone pre-treatment is required to establish infection with *C. trachomatis* by vaginal inoculation (15, 205, 210). Tuffrey and Taylor-Robinson suggested that the increased susceptibility caused by progesterone treatment in mice may be due to halting the shedding of epithelial cells during the natural cycle, thus increasing the number of target cells for chlamydial infection (205).

GONOCOCCAL HOST RESTRICTIONS

N. gonorrhoeae is highly adapted for survival inside its human hosts, to such a degree that Seifert put forth the hypothesis that both *N. gonorrhoeae* and *N. meningitidis* evolved from commensal species, rather than the reverse (176). Nearly all host restrictions for the gonococcus can be placed in three categories: colonization receptors, evasion of the host immune response, or obtaining nutrients from the host. Immune evasion mechanisms are many and varied and cannot be discussed without considering effective immune strategies; thus, these will be discussed below, in "Immunology of gonococcal and chlamydial infections."

Colonization receptors

N. gonorrhoeae can attach to host cells by many different mechanisms, many of which are either proven to be human-restricted or have not been tested in other animals. The initial interaction between *N. gonorrhoeae* and host epithelial cells is thought to be mediated by the type IV pilus. In primary human male urethral cells, this interaction involves binding to integrins; in primary human cervical epithelial cells, it takes place via a complex that forms between pilus, the inactivated cleavage product of complement protein C3b (iC3b), and the integrin CR3 (51). The major outer membrane porin PorB also forms a similar complex between iC3b and CR3 (51). In primary male urethral cells and primary endometrial cells and organ culture, LOS has also been shown to bind the asialoglycoprotein receptor, which may thus contribute to colonization of the female upper reproductive tract (51, 157, 203). Finally, some gonococcal Opa proteins can bind human carcinoembryonic antigen-related cell adhesion molecules (CEACAMs); other Opa variants bind heparin sulfate proteoglycans (51). The interactions between Opa

proteins and CEACAMs have been shown to be human-specific; transgenic mice expressing human CEACAMs have been successfully used to model *N. gonorrhoeae* infections and demonstrate the effect of certain Opa variants on colonization (84).

Nutritional factors

Unlike many other pathogens, *N. gonorrhoeae* does not produce any known siderophores, but can survive and thrive in a human host due to its ability to acquire essential nutrients such as iron and zinc from its host. Most metal acquisition systems utilize TonB-dependent transporters located in the neisserial outer membrane (138), expression of which is upregulated in response to metal deprivation (37). Thus far, receptor proteins specific for four different human metalloproteins have been identified (138). Three of these receptors allow *N. gonorrhoeae* to procure iron from transferrin, lactoferrin, and hemoglobin. The mechanisms for obtaining iron from transferrin and lactoferrin are similar, perhaps because the two metalloproteins are homologous with 60% sequence identity (212). Transferrin is an iron transport protein which binds Fe (III) and is found in serum as well as mucosal sites, where it also plays a role in host defense by restricting iron availability (138). Lactoferrin is also found in mucosal secretions (including breast milk, which is the source of the name), but is primarily in the cytoplasm of neutrophils. Lactoferrin has a binding affinity for iron approximately 300x that of transferrin and remains bound to iron at a pH of 3 or below (compared to transferrin, which releases Fe at a pH of 5) (173). Both transferrin and lactoferrin have bacteriostatic abilities, due in part to their ability to sequester free iron, thus depriving bacteria of iron and impeding bacterial growth (173). *N. meningitidis* and *N. gonorrhoeae* express two transferrin-binding proteins (Tbp) A and B in the outer membrane. These proteins form a

receptor that binds holo-transferrin with high affinity and removes the Fe(III) ion from the protein so that it can then be transported into the periplasm, after which apo-transferrin is released. Similarly, neisserial lactoferrin-binding proteins (Lbp) A and B bind lactoferrin and transport Fe(III) ions to the periplasm. Regardless of whether periplasmic Fe ions originated from lactoferrin or transferrin, a protein system consisting of Fe-binding proteins (Fbp) A B and C transports iron ions across the inner membrane and delivers them to the cytoplasm (138). Both the lactoferrin and transferrin-binding proteins in *Neisseria* are highly specific for the human proteins (102).

N. meningitidis and *N. gonorrhoeae* can also acquire hemoglobin by way of another TonB-dependent transporter and co-receptor, HpuB and HpuA (235). These systems remove heme from hemoglobin, after which heme is transported to the cytoplasm by an unknown mechanism, where iron is released from the heme in a degradation step catalyzed by HemO (235). *N. meningitidis* produces another hemoglobin receptor in addition to HpuAB called HmbR, which may reflect its greater proclivity to cause invasive disease. The use of hemoglobin, unlike transferrin and lactoferrin, is not host-restricted in the pathogenic *Neisseriae*, and an HmbR mutant strain was attenuated in an infant rat model (190).

Another essential nutrient that the pathogenic *Neisseriae* must acquire from their hosts is zinc. Like iron, zinc concentrations are tightly controlled by the innate immune system, particularly during infection. The neutrophil protein calprotectin plays an important role in this process. Calprotectin is released by neutrophils when forming neutrophil extracellular traps and sequesters zinc to prevent its use by invading bacteria (238). The human-specific neisserial species, however, have adapted to this defense

mechanism by evolving TonB-dependent transporters that bind calprotectin as a source of zinc. In *N. meningitidis*, this protein was originally named TdfH; after the discovery of its role as a receptor for calprotectin, it was renamed CP-binding protein A (CbpA) (191). *N. gonorrhoeae* has a homologous protein also called CbpA, which Jean *et al.* reported plays a role in zinc acquisition from calprotectin, thus conferring protection from neutrophil extracellular traps (87).

In addition to stealing nutrients directly from the human host, *N. gonorrhoeae* can also obtain essential nutrients from commensal bacteria by utilizing the iron-regulated outer membrane protein FetA (formerly FrpB) and the FbpABC iron acquisition system (18, 192). FetA scavenges xenosiderophores including ferric enterobactin in a TonB-dependent mechanism (18). Strange *et al.* showed that the FbpABC periplasmic iron transport system allowed Ton-independent acquisition of siderophores (192). It is not yet clear why utilization of xenosiderophores can be both Ton-dependent and Ton-independent; Strange hypothesized that expression levels of the proteins involved may determine which mechanism is the dominant one for individual strains (192).

The importance of gonococcal transferrin utilization in pathogenesis was demonstrated by studies showing that expression of either of the transferrin or lactoferrin receptors is required for infection of the human male urethra (8, 38). However, neither the transferrin or lactoferrin receptors are required for infection of the murine female reproductive tract, which might be predicted since *N. gonorrhoeae* can infect mice but cannot utilize murine homologs of these glycoproteins (91, 102). The demonstration that *N. gonorrhoeae* can grow *in vitro* in the presence of lactobaccilli unless an iron chelator is also present supports the hypothesis that *N. gonorrhoeae* scavenges iron from

commensal bacteria, including lactobacilli, in the female reproductive tract (91). Since then, we have shown that supplementation of hTf, although not required for infection of the murine LRT, is essential for supporting *N. gonorrhoeae* growth in the URT (155). We hypothesize that this requirement is due to the relatively low abundance of commensal bacteria in the URT, which forces *N. gonorrhoeae* to rely upon host iron sources.

IMMUNOLOGY OF GONOCOCCAL AND CHLAMYDIAL INFECTIONS

Immunology of the female reproductive tract

The female reproductive tract is an immensely complex system, and the barriers and responses to pathogens are equally complex. Here I have summarized existing data on the immunology of the female reproductive tract, focusing on aspects that are relevant to gonococcal and chlamydial infections. In many cases, the only available data focus on humans; in others, only animal models (primarily mice and rats). Unless otherwise stated, the data described below applies to humans.

The crucial challenge for the female reproductive tract is that the uterus must be an immunologically privileged site in order to avoid mounting an immune response to a developing fetus – but it must still maintain protection against any other invaders. Further complicating the issue is the constantly fluctuating balance of hormones and the associated changes in physical and chemical barriers to pathogens. The vaginal and ectocervical epithelia consist of stratified squamous cells, while the endocervix, uterine, and fallopian tube epithelia are made of columnar epithelial cells with tight junctions (Fig. 2). The tightness of the junctions seems to be affected by estradiol, which decreases transepithelial resistance in murine epithelial cells; progesterone had no effect (224). Mucus coating the epithelium provides an additional physical as well as chemical

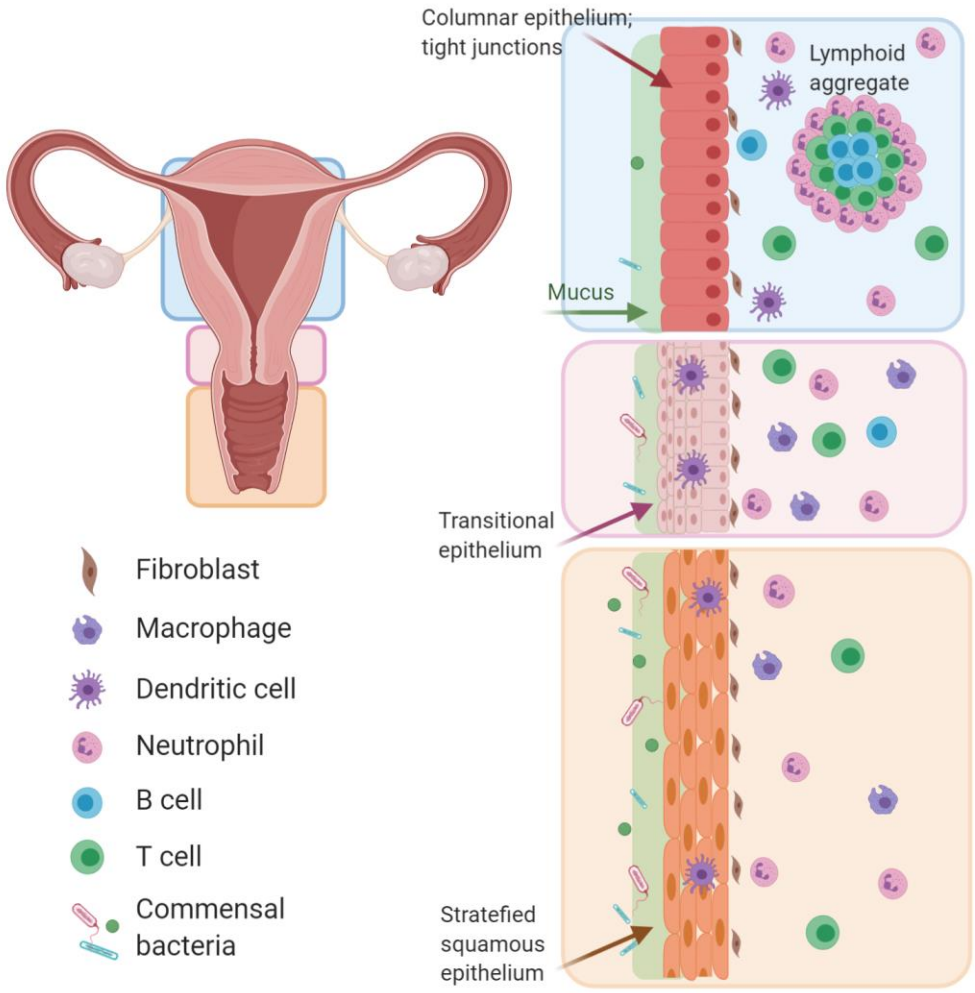

Figure 2. Immunology of the female reproductive tract

Different anatomical regions of the female reproductive tract differ in many important ways, including the structure of the epithelium, the relative abundance of cell types, and commensal bacteria. Information used to make this figure was from (223, 225); figure made using Biorender.com

barrier, as it is acidic (normal pH around 4 – 5) and contains complement and antimicrobial peptides produced by epithelial cells. The consistency of mucus depends upon ovarian hormone levels: during estrogen-dominant stages, it is thin and watery to allow sperm movement; during progesterone-dominant stages, it is thick and sticky (73).

The number and proportions of phagocytic and antigen-presenting cells depends upon the location within the reproductive tract as well as the stage of the hormonal cycle (Fig. 2). Neutrophils are the most prevalent immune cell in the reproductive tract, with numbers strongly correlated with the menstrual cycle and peaking during menses, probably to assist in breaking down endometrial tissue and to provide additional defense while the epithelial barrier is disrupted. Macrophages in the uterus are primarily in the stroma, with highest numbers just before menses; in the LRT, macrophage numbers are stable throughout the menstrual cycle. Dendritic cells are also located primarily in the stroma of the uterus and are sensitive to as-yet unidentified soluble mediators secreted by epithelial cells that induce a tolerogenic phenotype and decreased sensitivity to Toll-like receptor (TLR) stimulation (73).

In addition to antigen presentation by professional antigen presenting cells, epithelial cells in the reproductive tract can also process and present antigen, and this process is also controlled by ovarian hormones (224). The hormonal effects were further explored by Wira and colleagues, who examined antigen presentation in rats and mice (226). When isolated from rats treated with estradiol, uterine epithelial cells up-regulated antigen presentation compared to those from untreated animals; however, under the same conditions, uterine and vaginal stromal cells had decreased antigen presentation, and this was at least partially mediated by transforming growth factor β (TGF-β) secreted by

epithelial cells (226). Puzzlingly, and in contrast to rats, the effect of estradiol on antigen presentation by epithelial cells was reversed in mice (224).

The expression of pattern recognition receptors (PRRs) in the reproductive tract has been the subject of much debate and many conflicting studies. TLR-4 recognizes lipopolysaccharide (LPS) and is important for detection of Gram-negative bacteria; this receptor has been shown to be expressed on primary uterine epithelial cells and upregulated in response to intra-uterine inflammation in rats (224). Moreover, TLR-deficient mice do not develop endometritis in response to exposure to LPS (178). However, expression of TLR-4 in other regions of the genital tract is more controversial: a number of studies have been unable to detect expression in the vagina and ectocervix, with the exception of one; reports on the fallopian tubes are also conflicting. The reproductive tracts of mice do express TLRs 1-9, and murine uterine epithelial cells were responsive to stimulation by ligands of TLR 1-6 (224). Expression of TLRs is also regulated by ovarian hormones: TLRs 2 – 6, 9, and 10 were shown to have decreased expression in endometrial tissue during the menstrual and proliferative phases, suggesting inhibition by estradiol and/or promotion by progesterone (1).

Secretion of proinflammatory cytokines and chemokines in humans is also affected by estrogen and progesterone levels. Expression of interleukin (IL)-1α is highest in the endometrium in the secretory phase and drops markedly in the proliferative phase when estradiol levels are highest (197). Similarly, tumor necrosis factor α (TNF-α), IL-1β, IL-6, and IL-8 were all found to have lower expression levels during the proliferative and early secretory phases, with a peak in the late secretory stage (209). Likewise, in

mice, estradiol treatment decreases uterine epithelium secretion of TNF-α, which plays a prominent role in inflammation, and also inhibits secretion of IL-1β (224) (Wira 2005).

T lymphocytes are the most abundant immune cells in the reproductive tract, and are more prevalent in the URT compared to the LRT (225). Within the population of T cells, CD8 T cells outnumber CD4 T cells by about 1.5 – 2-fold. Lymphoid aggregates are unique structures only found in the uterus, consisting of a core of B cells, surrounded by CD8 T cells, with an outer ring of macrophages. These structures are largest during the secretory phase of the menstrual cycle, smaller during the proliferative phase, and completely absent in post-menopausal women, suggesting that their formation is dependent upon ovarian hormones (73). The purpose of these lymphoid aggregates is unknown, but based on the timing of their formation within the menstrual cycle, it has been suggested that their purpose is to suppress T cell immunity that might otherwise harm an embryo in the process of implantation (73). Both immunoglobulin (Ig) A and IgG play important roles in protection of the reproductive tract, and here too secretion is hormonally regulated. The epithelial cell receptor pIgR is responsible for transporting IgA from tissue to lumen; expression of this receptor is highest during the secretory phase, significantly decreased during the proliferative phase, and lowest during menses. Meanwhile, IgG in the uterine mucosa is highest near ovulation, but lowest in the fallopian tubes. The LRT follows a similar pattern, with the lowest levels of IgG and IgA around the time of ovulation, gradually rising until just before menses (221).

N. gonorrhoeae host-pathogen interactions in genital tract infections

The initial immune response to *N. gonorrhoeae* infection involves acute inflammation, which is likely due to signaling through TLR-4, which recognizes

gonococcal LOS (107). Other bacterial elements that may play a role in stimulating inflammation include outer membrane blebs, release of genomic DNA, peptidoglycan fragments, and heptose phosphates (172). Due to any or all of these factors, Th17 cytokines including IL-17 and IL-23, along with several other pro-inflammatory cytokines, have been shown to be elevated in serum and genital secretions from infected men and women (113). Secretion of these cytokines results in rapid recruitment of neutrophils and macrophages to the site of infection.

N. gonorrhoeae is particularly adept at surviving in and around neutrophils; the bacteria can survive phagocytosis and even replicate inside the phagosome due to prevention of phagosome maturation by mechanisms that are not yet understood (148). Degranulation of neutrophils releases a number of bactericidal components, including cationic antimicrobial peptides (CAMPs). *N. gonorrhoeae* strains have varying susceptibility to CAMPs due to several different mechanisms that confer resistance to CAMPs. These mechanisms include over-expression of the MtrCDE active efflux pump and modification of Lipid A to decrease charge-dependent binding of CAMPs. Neutrophil extracellular traps (NETs) are another mechanism by which neutrophils capture and kill pathogens; in gonococcal infections, this is prevented by secretion of the highly conserved thermonuclease Nuc, which degrades NETs. However, efficient neutrophil killing of *N. gonorrhoeae* does occur under one condition. When *N. gonorrhoeae* expresses Opa protein variants that bind CEACAM3, which is expressed only on neutrophils, a proinflammatory signaling cascade is triggered that results in opsonin-independent phagocytosis and an oxidative burst that kills the bacteria (148).

N. gonorrhoeae interactions with macrophages are not as well defined, and there were conflicting reports regarding the fate of macrophage-phagocytosed *N. gonorrhoeae* in the 1970s and 1980s (30). More recently, however, Leuzzi and colleagues reported that phagocytosis results in death for most *N. gonorrhoeae*, but some bacteria can survive intracellularly for at least 24 hours; Zughaeir and colleagues reported similar findings (105, 237). Chateau and Seifert demonstrated that not only can a subset of *N. gonorrhoeae* survive and replicate after phagocytosis, but the remaining bacteria modulate macrophage apoptosis and cytokine secretion (30). This finding is consistent with the findings of Ortiz *et al.* who found that stimulation with *N. gonorrhoeae* resulted in an alternative M2 phenotype; these macrophages were also unable to stimulate T cell proliferation and upregulated expression of programmed death ligand 1 (146).

The complement system is another part of the first line of defense, and evasion of complement-mediated killing is important for *N. gonorrhoeae* to survive at the cervical epithelium and in the bloodstream (50, 141). *N. gonorrhoeae* is highly adapted to subvert and evade human complement. There are two main ways in which *N. gonorrhoeae* prevents complement-mediated destruction: inactivation of complement components, and binding to regulatory components in order to present as "self". *N. gonorrhoeae* can bind CR3, C3B, and the receptor for iC3B, all of which result in destruction of the individual component or prevent activation (157). Whether or not these activities are specific to human complement proteins is unknown; however, the fact that *N. gonorrhoeae* is susceptible to killing by serum from other animals suggests that it is human specific. Complement-mediated killing is further decreased by *N. gonorrhoeae* binding to human

Factor H and C4b-binding protein, both of which serve as complement inhibitors when bound to cell surfaces (172).

Natural infection with *N. gonorrhoeae* does not induce a protective immune response, and reinfection is common. Phase and antigenic variation are a contributor to the ability of the gonococcus to evade a specific antibody response. Several major surface structures of *N. gonorrhoeae* are antigenically or phase variable. Antigenic variation is primarily responsible for the number of different variants of Type IV pili that can be expressed. The major subunit of the Type IV pilus within expressed pili is PilE ("expressed"); however, there are also approximately 20 additional copies of storage loci (*pilS*), that encode partial "silent" variants of PilE, all of which can be recombined in and out of the expression locus (175).

In addition to this process of pilin antigenic variation, many neisserial outer membrane proteins undergo phase variation, which allows expression of the protein to be switched on or off. The list of phase-variable proteins in *N. gonorrhoeae* is long, but notably includes the Opa proteins, which bind to several different human CEACAMs and allow bacterial adhesion. Most strains of *N. gonorrhoeae* have 11 − 12 different *opa* genes, each of which is independently phase-varied. Additionally, *opa* genes can recombine with each other, resulting in the generation of new *opa* alleles; hence, hundreds of *opa* alleles have been identified to date. Similarly, the LOS structure of *N. gonorrhoeae* can also be altered by the phase-variable expression of glycotransferase enzymes responsible for attaching glycan side-chains to LOS (170).

Humoral immunity to *N. gonorrhoeae* infection is limited. Evidence from human studies examining the antibody response to whole-cell *N. gonorrhoeae* demonstrate only

a slight increase in *N. gonorrhoeae*-specific IgA and IgM in infected patients compared to uninfected patients, perhaps due to the confounding factor of commensal *Neisseria* species, or exposure to *N. meningitidis* (113). Other studies have found small but significant increases in antibodies for specific antigens, including pilus, LOS, porin, transferrin binding proteins, and other outer membrane proteins (113). The finding that antibodies to the outer membrane protein Rmp (reduction modifiable protein) can block binding of antibodies to other epitopes may help explain why none of these result in protection from reinfection (172). Additionally, *N. gonorrhoeae* possesses a highly conserved IgA protease that is homologous to the meningococcal IgA protease, which has been shown to generate Fab fragments that block binding by functional antibodies. This protease is highly conserved, suggesting that it has an important function, but its role in evading the antibody response has not yet been established (172).

There is some evidence that natural infection with *N. gonorrhoeae* produces a cellular immune response, although this evidence is mainly limited to measuring lymphocyte proliferation upon exposure to *N. gonorrhoeae* antigens (113). Analysis of cytokines in serum and genital secretions from *N. gonorrhoeae*-infected patients suggests that the T cell response is skewed toward a Th17 type response, consistent with the acute inflammation seen in *N. gonorrhoeae* infections (172). Similar findings in the estradiol-treated mouse model of infection confirm the induction of the Th17 pathway by *N. gonorrhoeae*, although there is somewhat conflicting evidence regarding the role that the Th17 response plays in protection (53, 54). When IL-17 activity is blocked, either by IL-17 depletion or use of IL-17 receptor knock-out mice, *N. gonorrhoeae* infection is prolonged (53). However, depletion of IL-22 (another cytokine involved in Th17

responses) results in more rapid clearance of infection (54). It is not yet clear how or why these two cytokines have apparently opposing roles in gonococcal infection clearance. Further studies explored the role of TGF-β in the development of T cell responses to infection. TGF-β is a cytokine that plays an important role in T cell differentiation and tolerance to self-antigens and is upregulated during *N. gonorrhoeae* infection of mice (109). Blocking TGF-β during *N. gonorrhoeae* infection altered the T cell response from a Th17-dominant response to a Th1/Th2 response and led to faster clearance of infection and protection to secondary infection (112). The protective response was shown to be due to Th1 cells, as in the absence of IFNγ signaling, protection did not occur. Further support for the protective role of Th1 responses comes from mouse studies during which microencapsulated IL-12 was administered during infection; this resulted in development of Th1 and humoral immunity and a memory response (106) that caused more rapid infection clearance upon reinfection with the same *N. gonorrhoeae* strain as well as a heterologous strain (106, 108, 111).

Reports that *N. gonorrhoeae* infection induces regulatory T cell responses also provide potential explanations for the lack of protective immunity from natural infections. Imarai and colleagues report a significant increase in FOXP3-positive regulatory T cells (Tregs) in *N. gonorrhoeae*-infected mice (83). In contrast, Liu and colleagues found no increase in FOXP3-positive Tregs in mice but did detect an increase in a different type of regulatory T cells called Tr1 cells, which are FOXP3-negative (110). Tr1 cells are characterized by expression of the immunosuppressive cytokine IL-10 and are produced when naïve CD4 T cells are stimulated by tolerogenic dendritic cells in the presence of IL-10. Blockage of IL-10 was found to enhance Th1, Th2, and Th17

responses during gonococcal infection, and resulted in faster infection clearance as well as protection against reinfection (110).

C. trachomatis host-pathogen interactions in genital tract infections

Initial recognition of *C. trachomatis* is probably also through stimulation of TLRs, but which specific TLRs are responsible is not entirely clear. Taylor and colleagues reported that variations in genes for TLR2 and TLR6 were associated with increased inflammation in women with PID (200). Agrawal reported that TLR2 and TLR4 are both upregulated in human cervical cells during chlamydial infection (3). TLR2 signaling is an important component of the response to *C. muridarum* in mice and is associated with increased oviduct pathology; no such relationship was found for TLR4, however (47). Additionally, the cytosolic receptor Nod1 has been reported to detect chlamydial peptidoglycan during infections with both *C. trachomatis* and *C. muridarum*; absence of Nod1 significantly reduces expression of pro-inflammatory genes (213).

Chlamydial LPS is about 100-fold less inflammatory than gonococcal LOS (32). Chlamydial species have also been shown to interfere with apoptosis of host cells. There are conflicting reports on whether apoptosis is suppressed or promoted during infection; a likely explanation is that apoptosis is inhibited early in the developmental cycle and promoted near the end of the cycle (32). This effect may also differ depending on the cell type infected and the chlamydial strain.

Another important difference between *C. trachomatis* and *N. gonorrhoeae* infections is that natural infection with *C. trachomatis* does seem to confer some immunity to reinfection, albeit short-lived and not completely protective (58). Studies in animal models have explored this question further. Mice and guinea pigs develop

antibody and cell-mediated immunity within about 10-14 days of infection; antibiotic treatment before 10 days of infection prevents the development of protective immunity (164). Complete protection from reinfection lasts for about 42- 60 days in mice, and about 30 days in guinea pigs. Partial immunity, consisting of a reduction in bacterial burden, shorter infection course, or reduction of oviduct pathology, lasts for approximately 200 days in mice and over two years in guinea pigs. The source of this protection has been the subject of some debate, partly due to differences in results from different animal models. Antibodies are essential for protection in guinea pigs, but B cell knock-out mice are resistant to reinfection unless CD4 cells are also depleted (164). The exact mechanism of antibody-enhanced infection clearance has not been definitively shown; antibodies may function by blocking EB entry to cells, by enhancing phagocytosis, or a combination of the two. In contrast, cell-mediated immunity is essential in both mice and guinea pigs, and CD4 T cell counts directly correspond to protection from reinfection in mice. One proposed paradigm for the interplay between the two forms of immunity is that antibodies are responsible for an initial reduction in bacterial burden, while T cells are necessary for actual clearance of infection (164).

Interferon γ (IFN-γ) plays an important role in the immune response to both *C. muridarum* and *C. trachomatis*, although the mechanism is different for the two species as discussed above in "Chlamydial animal models". Early in infection, natural killer cells are the primary producer of IFN-γ; later, Th1 cells contribute. However, Zhang and colleagues showed that too much IFN-γ and IL-12 can prevent development of memory CD8 cells; depleting IFN-γ led to a stronger memory response and enhanced infection clearance in mice (233).

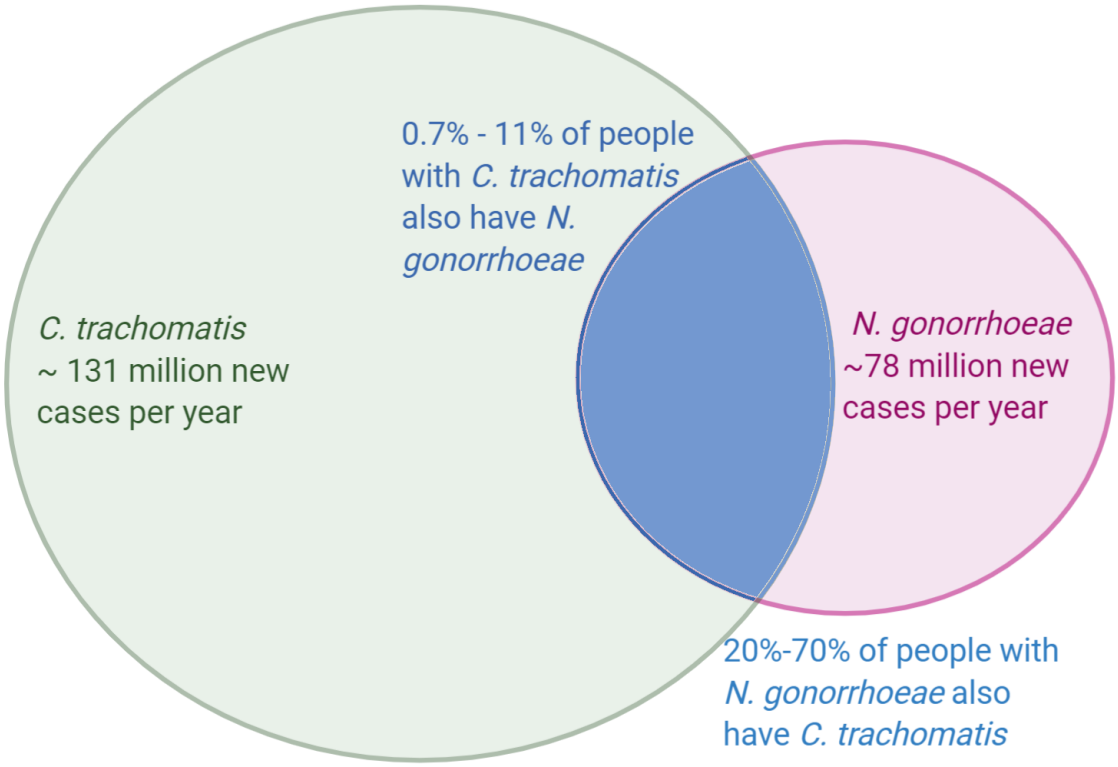

Figure 3. *N. gonorrhoeae*/*C. trachomatis* coinfections as a proportion of total cases of gonorrhea and chlamydia
Shown are the total estimated number of *C. trachomatis* (green) and *N. gonorrhoeae* (pink) new infections per year (worldwide) as reported by Newman (140). The estimated number of coinfections ranges widely (49, 123, 142), and coinfections represent a higher proportion of all cases of gonorrhea than chlamydia, due in part to the higher prevalence of *C. trachomatis*.

GONOCOCCAL/CHLAMYDIAL COINFECTIONS

Concurrent *N. gonorrhoeae* and *C. trachomatis* are common, particularly in adolescent and young adult populations (Fig. 3). The percent of *N. gonorrhoeae* infections with concurrent *C. trachomatis* infections in this age range has variously been reported as 42.7%, 49%, and 70% (49, 123, 142). The actual percentage is likely highly dependent on age and other risk factors for sexually transmitted infections. The proportion of *C. trachomatis* patients with a gonococcal coinfection is usually lower than the reverse, probably due to the overall higher prevalence of *C. trachomatis*, with published estimates ranging from 0.7% to 11% (123, 142). There is some evidence, though sparse, that chlamydial coinfection may have an impact on *N. gonorrhoeae* infection. Stupiansky and colleagues reported in 2011 that adolescent women with *N. gonorrhoeae*/*C. trachomatis* coinfections had evidence of increased *N. gonorrhoeae* bacterial burden compared to women with *N. gonorrhoeae* alone; however, the sample size was small, and this difference was statistically insignificant (193).

Any differences in the immune response to coinfections could be especially important when considering harmful complications of *N. gonorrhoeae* and *C. trachomatis* infections such as PID and associated sequelae, particularly for vaccine development. Possible evidence for an altered immune response to coinfection comes from a retrospective case/control study, which found that the meningococcal vaccine Men4CB reduced the incidence of gonococcal infection; however, the apparent effectiveness of the meningococcal vaccine was halved when chlamydial coinfection was also present (154). This study was conducted by identifying patients with either gonorrhea (cases) or chlamydia (controls) and determining whether they had previously received the Men4CB vaccine. These researchers found that patients with gonorrhea were

31% less likely to have received the vaccine than patients with chlamydia. Intriguingly, patients with gonorrhea/chlamydia coinfection were only about 14% less likely to have been vaccinated than those with chlamydia alone, indicating that vaccine effectiveness was approximately halved for people with both gonorrhea and chlamydia compared to those with gonorrhea alone. Whether this finding reflects an actual difference in the vaccine-induced memory response in the context of a coinfection, or whether chlamydial infection may somehow reduce vaccine efficacy against gonorrhea by some other mechanism, remains to be determined.

Finally, another major problem confronting the field of STI research is the ever-increasing antibiotic resistance of *N. gonorrhoeae* and the expense and need to treat for *C. trachomatis* coinfection empirically in individuals with gonorrhea. The emergence of resistance to first the fluoroquinolones and then the extended-spectrum cephalosporins in the gonococcus has led to a dearth of effective antibiotics against *N. gonorrhoeae* (206). New antibiotics are desperately needed, and ideally these would be effective against both *N. gonorrhoeae* and *C. trachomatis*, either separately or in coinfected individuals (6). Dually-active antibiotics would simplify treatment, particularly in regions where access to testing is limited and treatment is often empiric (6). In order to develop and test such antibiotics, animal models in which treatment efficacy against both pathogens can be tested simultaneously are needed. Moreover, the prevalence of URT infections due to these pathogens creates a need for testing the efficacy of antibiotics in the URT.

Gonorrhea/chlamydia coinfection model

In 2011, our lab published a novel method for establishing gonococcal/chlamydial coinfection in mice (210). This coinfection model relied upon the use of *C. muridarum* as

a surrogate model for chlamydial genital infection in mice, combined with the use of the estradiol-treated mouse model for gonococcal genital infection. The primary obstacle to establishing *N. gonorrhoeae*/*C. muridarum* coinfection in mice is that mice are susceptible to the two pathogens in different stages of the estrous cycle, with maximum susceptibility to *C. muridarum* in progesterone-dominant phases of the estrus cycle, peaking in diestrus (15, 205, 210); at the same time, mice only remain infected with *N. gonorrhoeae* while estradiol-treated or in estradiol-dominant phases of the estrous cycle (92) (discussed in more detail in "Animal models: Hormonal cycles" above). In order to overcome this obstacle, Vonck developed a method in which mice were inoculated on three consecutive days with *C. muridarum* (assuming that on at least one of those days, mice would be in diestrus and thus susceptible to *C. muridarum*). Several days after inoculation with *C. muridarum*, mice were swabbed to identify mice in the diestrus phase of the estrus cycle, after which estradiol treatment was initiated; mice were then inoculated with *N. gonorrhoeae* two days later as in the previously established estradiol-treated mouse model. By staggering the inoculation in this way, mice could be coinfected with *C. muridarum* and *N. gonorrhoeae*. Using this model, Vonck conducted basic characterization of murine coinfection with *N. gonorrhoeae* and *C. muridarum*. Her primary findings were that coinfected mice had a markedly higher local inflammatory response as shown by increased PMN influx into vaginal tissue as well as increased levels of local proinflammatory cytokines and chemokines. Curiously, coinfected mice also exhibited higher levels of *N. gonorrhoeae* colonization than mice infected with *N. gonorrhoeae* alone, while *C. muridarum* colonization seemed to be unaffected by the presence or absence of *N. gonorrhoeae*; these findings were reminiscent of those reported

in the natural history study on gonorrhea, chlamydia, and gonorrhea/chlamydia coinfection described above (193). The increase in *N. gonorrhoeae* colonization became more apparent the longer mice were infected with *C. muridarum* prior to *N. gonorrhoeae* infection.

PURPOSE OF THIS WORK

The development of the hTf-supplemented mouse model of *N. gonorrhoeae* URT infection has opened the door to many possible advancements in the field. Many questions about the model and gonococcal PID remain to be answered, which I have begun to address in the research described here. Some of these questions include: (1) Does the immune response in the hTf mouse model resemble that in human infections? (2) How does the immune response to naturally ascending infection differ from infections resulting from direct inoculation into the uterus, and which of these is the better strategy for future work using the model? (3) Do previous findings regarding the suppressed immune response in the LRT infection mouse model hold true for the hTf-supplemented mouse model? Furthermore, although the previous model of gonococcal/chlamydial coinfection developed by Vonck et al. (210) has proved useful, two major drawbacks to the model are that (1) coinfection is limited to the LRT, as in the estradiol-treated model of *N. gonorrhoeae* infection; and (2) the model depends on inoculation of *C. muridarum* first, followed by *N. gonorrhoeae*, which only represents one possible scenario by which coinfection may be established. With the development of the human-transferrin supplemented model of *N. gonorrhoeae* URT infection, I set out to expand this model for the purpose of studying gonococcal/chlamydial URT coinfections.

CHAPTER 2: Route of inoculation and bacterial burden influence the immune response in a mouse model of *Neisseria gonorrhoeae* upper reproductive tract infection

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***To be submitted pending histopathology studies**

The work presented here is the sole work of Claire Costenoble-Caherty, with the following exception: A. Macintyre performed cytokine measurements.

ABSTRACT

Pelvic Inflammatory Disease is a serious condition that can result from untreated *Neisseria gonorrhoeae* genital tract infections ascending to the uterus and Fallopian tubes. Recently, we reported that human transferrin (hTf) supplementation of mice allows sustained upper reproductive tract infection in mice. Here we have defined the local cytokine and CD4 T cell response to *N. gonorrhoeae* using the newly established hTf-supplemented mouse model and explored whether naturally ascending infection following vaginal inoculation differs from direct transcervical inoculation in immunologically relevant aspects. We demonstrated a local pro-inflammatory cytokine response to infection that is highly dependent on bacterial burden in vaginally-inoculated mice. Decreased Th1 and Th2 cells were associated with increased bacterial burden in the lower reproductive tract, while lower numbers of regulatory T cells were associated with a higher bacterial burden in the upper reproductive tract. Depletion of transforming growth factor β (TGF-β), previously shown to induce a protective Th1 response and rapid clearance of *N. gonorrhoeae* infection, caused infection clearance in vaginally-inoculated mice, although transferrin supplementation reduced this effect. Conversely, TGF-β

depletion had no effect on clearance of *N. gonorrhoeae* infection in transcervically-inoculated mice. Together, these results suggest that the immune response to *N. gonorrhoeae* infection is strongly dependent on the bacterial burden and location of bacteria in the reproductive tract. These data demonstrate that *N. gonorrhoeae*-induced signaling through immunological pathways appears to differ with respect to these two compartments of the female reproductive tract and provide important context for future studies utilizing the model.

INTRODUCTION

Neisseria gonorrhoeae is the causative agent of gonorrhea, which is the second most common bacterial sexually transmitted infection world-wide. In women, gonorrhea usually manifests as cervicitis; however, 15-20% of untreated infections can ascend to the upper reproductive tract (URT), which can result in Pelvic Inflammatory Disease (PID). PID is a syndrome defined as inflammation of the URT, including the uterus, fallopian tubes, and ovaries, due to an infection (22). Approximately 85% of cases of PID are caused by sexually transmitted pathogens, most commonly *Chlamydia trachomatis*, *N. gonorrhoeae* and *Mycoplasma genitalium* (22). PID may also be caused by anaerobic bacteria that are commonly found in the vaginal microbiome, such as *Gardnerella vaginalis* and *Prevotella* spp. (67, 75). Due to the difficulty of sampling the URT, the causative organism is often presumptively identified based on its detection in cervical or vaginal swabs by culture and/or nucleic acid-based diagnostic tests; in about 25-30% of cases the causative organism cannot be identified (29, 122).

PID can be silent or acute and may present in a variety of ways. Tubo-ovarian abscesses are one of the most severe manifestations of PID and are often accompanied by

severe abdominal pain as well as systemic symptoms including fever and vomiting (12). Less severe forms of PID are characterized by mild to moderate abdominal and/or pelvic pain and abnormal vaginal discharge. When untreated, women with PID may subsequently suffer from chronic pain, ectopic pregnancy, and infertility. A longitudinal cohort study carried out in Sweden between 1960 and 1984 assessed the risk of these conditions following a diagnosis of PID, and found that after a single episode, approximately 18% of women suffered from chronic pelvic pain and 8% of women experienced tubal factor infertility; the rate of infertility increased to ~40% after 3 or more episodes (216). The risk of ectopic pregnancy increased approximately 6-fold with the first episode of PID; 9.1% of first pregnancies were ectopic in women with laparoscopically confirmed salpingitis, compared to 1.4% of pregnancies in women without salpingitis.

Due to a combination of factors, including the number of asymptomatic infections, the variety of different organisms that are associated with PID, and the inability to sample the URT without invasive procedures such as endometrial biopsy, there are many unresolved questions surrounding PID. Animal models of female URT infections can provide useful systems for studying immune responses as well as microbial factors that may affect disease progression and pathology. Until recently, chlamydial infections have been the focus of PID animal research due to the wider availability of animal models. Animal models for chlamydial PID include *Chlamydia muridarum* and *Chlamydia caviae* infections in mice and guinea pigs, respectively, which are the natural hosts of these species, and the use of macaques and mice to study *C. trachomatis* (16, 48, 127, 132). A murine model of *M. genitalium* was also developed, in which mice vaginally

inoculated with *M. genitalium* remained colonized in the lower reproductive tract (LRT) for up to 11 weeks (118). Ascension of *M. genitalium* to the URT and hydrosalpinx formation were observed in 65% and 57% of estradiol-treated, *M. genitalium*-infected mice, respectively.

As *N. gonorrhoeae* is a human-only pathogen, and no other neisserial species have been identified to date that could serve as a surrogate model for gonococcal genital tract infections, it has proved to be much more difficult to develop animal models of *N. gonorrhoeae* infections. *N. gonorrhoeae* LRT infection can be established in estradiol-treated mice, and this model has been used for a broad range of applications, including identification of virulence factors (57, 77, 92, 171, 236) characterization of the immune response to infection (53, 110, 112, 147, 234), testing of candidate antibiotics (26, 36) and microbicides (188), and vaccine testing (63, 108). In this model, gonococci are found in the vaginal lumen, are associated with vaginal and cervical tissue, and are seen within the lamina propria (187). When this model is used with BALB/c mice, a proinflammatory cytokine and chemokine response and a vaginal PMN influx occur in response to infection (147). Gonococcal replication occurs during infection despite the absence of hTf or lactoferrin as usable iron sources (91). In this model, however, only about 10-15% of mice exhibit ascending infection, which is transient and is associated with only low numbers of gonococci recovered from the endometrium (92). Thus, previous studies attempting to gain a better understanding of *N. gonorrhoeae*-induced PID have primarily used human tissue and epithelial cell lines. The fallopian tube organ culture model has been used to identify *N. gonorrhoeae* virulence factors, including lipooligosaccharide and peptidoglycan fragments, which have toxic effects on ciliated cells that are mediated by

TNF-α, and to define the cytokine and chemokine response to *N. gonorrhoeae* infection (104, 114). Other studies have utilized three-dimensional endometrial epithelial cell models; these cultured tissue models have yielded invaluable insights on neisserial interactions with human cells, including identification of adhesion factors and the endometrial cytokine response to infection (72, 100, 101)

Despite the value of these organ and cell culture model systems, the lack of whole animal models has been a hindrance to determining how *N. gonorrhoeae* infections of the URT may differ from those confined to the LRT. There are several facets of reproductive tract infections that are difficult to study without animal models, including long-term effects on fertility, systemic immune responses, and potential differences in vaccine and antibiotic efficacy against infections of the LRT vs the URT. This is partly because the female reproductive tract is an incredibly complex system with many elements, including hormonal influences, that have yet to be fully characterized. The URT is immunologically distinct from the LRT because the two sites fulfill very different roles. The LRT is colonized by abundant bacteria and has been the subject of numerous studies; in contrast the URT, previously thought to be sterile in healthy women, contains a lower bacterial biomass but more diverse species (2, 7). The endometrium, despite the requirement for immunological tolerance of the fetus, possesses higher numbers of leukocytes per gram of tissue than other sites in the reproductive tract. The exact composition of this leukocyte population has been the subject of conflicting reports, although it seems to be dominated by natural killer cells, macrophages and dendritic cells, neutrophils, and T cells (223, 226). Interestingly, few B cells are present at any of the anatomical sites within the reproductive tract (73, 222, 225). The distribution of

pattern recognition receptors (PRRs) also differs with respect to the location within the reproductive tract. Toll-like receptor (TLR)-4, which recognizes lipopolysaccharide, is expressed on uterine epithelial cells and is upregulated during inflammation; expression of TLR-4 in other regions of the reproductive tract is less certain, with conflicting reports regarding expression in the vagina, ectocervix, and fallopian tubes (224). TLR-9, which recognizes microbial CpG DNA, is present and functional throughout the female reproductive tract (68, 156). TLR-10, on the other hand, which recognizes an as-yet undetermined ligand and produces an anti-inflammatory response, is expressed only in Fallopian tube cells (68). Expression of several of these receptors varies due to hormonal influences throughout the menstrual cycle; during menses and the proliferative phase, TLRs 2-6, 9, and 10 have decreased expression in endometrial tissue (1). It follows, therefore, that the immune response to invading pathogens may be dictated by the location of the infection and the timing of infection with respect to the menstrual cycle.

Several recently published studies have yielded promising opportunities for advancement of animal modeling of gonococcal PID. In 2016, Islam *et al.* described a model in which diestrus-stage or progesterone-treated mice that are transcervically inoculated with high doses of *N. gonorrhoeae* exhibit a rapid inflammatory response similar to that of symptomatic PID in humans (55, 85). The effect of hormonal state on the responsiveness of URT tissue to *N. gonorrhoeae* was also examined; however, one drawback to this model is that the recovery of *N. gonorrhoeae* rapidly declined during the first 48 hours post-inoculation and no live organisms were recovered after 72 hours post-inoculation. More recently, we described a modification of the estradiol-treated mouse model of *N. gonorrhoeae* infection in which we utilize hTf supplementation to support *N.*

gonorrhoeae infection of the URT (155). Human transferrin, a host-restricted iron source for *N. gonorrhoeae*, boosts the vaginal bioburden, and high numbers of viable gonococci are recovered from the endometrium for at least 7 days following transcervical inoculation with *N. gonorrhoeae* in approximately 75% of mice. *N. gonorrhoeae* is also recovered from the oviducts in 28-57% of mice. Ascending infection following vaginal inoculation also occurs in about 70% of mice.

The hTf-supplemented mouse model provides a system for examining host responses to *N. gonorrhoeae* in the URT over the course of infection. A vaginal polymorphonuclear leukocyte (PMN) response to infection is detected in hTf-treated mice given Premarin to promote susceptibility to *N. gonorrhoeae* (155), which does not cause high sustained levels of plasma 17β-estradiol (E. L. Raterman *et al.*, submitted). The immune response in the URT in this model has not yet been examined. Pathology due to PID is primarily mediated by the immune response to infection and subsequent tissue damage and scarring, and the lack of an intact immune system is one of the drawbacks of tissue and organ culture models.

The objective of the current study was to define the murine innate and adaptive immune responses to *N. gonorrhoeae* infection in the hTf-supplemented mouse model. By doing so, we hope to gain an understanding of host responses to *N. gonorrhoeae* in the URT and how these responses compare to what is known about the immune response to *N. gonorrhoeae* URT infections in the fallopian tube organ culture and endometrial cell culture systems, and in humans.

METHODS

Bacterial strains and culture conditions

N. gonorrhoeae strain FA1090, which was originally isolated from a patient with disseminated gonococcal infection and has been well-characterized in human volunteers, was used in this study (35, 78). *N. gonorrhoeae* was cultured on GC agar (BD Biosciences) supplemented with Kellogg's supplement and 12 μM $Fe(NO_3)_3$ under 7% CO_2 at 37°C. For isolation of *N. gonorrhoeae* from mice, supplemented GC agar with vancomycin, colistin, nystatin, trimethoprim, and streptomycin (GC-VCNTS agar) was used (88).

Animal use assurances

All animal experiments were conducted at the Uniformed Services University of the Health Sciences (USUHS), a facility fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, under a protocol that was approved by the USUHS Institutional Animal Care and Use Committee in accordance with all applicable federal regulations governing the protection of animals in research.

Experimental murine infection

Female BALB/c mice (NCI BALB/c strain; 6 to 7 weeks old; Charles River Laboratories) were used for all experiments. Mice were housed, food and water ad libitum, with 12-hour light/dark cycles. Mice were infected with *N. gonorrhoeae* as described (165). Briefly, two days prior to inoculation with *N. gonorrhoeae*, mice in the diestrus or anestrus phases of the estrus cycle were identified by cytological examination of vaginal swab smears, and then given 0.5 g of Premarin via subcutaneous injection (Day -2). Mice were also treated with antibiotics to reduce the overgrowth of commensal flora that occurs under the influence of estradiol as described (165). A second dose of 0.5 g of Premarin was administered two days later (day 0), four hours prior to bacterial

inoculation, and a third dose was given two days after inoculation (day +2). On day 0 through the study endpoint, mice were given 8 mg of human transferrin, dissolved in sterile endotoxin-free PBS via intraperitoneal injection. Mice were inoculated either transcervically or vaginally on day 0 (1.5-2 hours post-hTf injection) with 10^6 colony-forming units (CFU) of *N. gonorrhoeae* suspended in 20 uL of endotoxin-free PBS. Transcervical inoculations were performed using flexible tubing from a 26-gauge intravenous catheter attached to a 200 uL pipette tip. Mock-inoculated control mice were inoculated in an identical manner, using sterile endotoxin-free PBS. Vaginal inoculations were administered with a 200 uL pipette tip inserted into the vagina. In all experiments, the remaining inoculum suspensions were quantitatively cultured for *N. gonorrhoeae* to verify infectious dose.

Infection of the LRT was measured by culturing vaginal swabs, which were collected by inserting a moistened sterile Puritan rayon swab (Fisher Scientific) into the vagina. A small portion of the vaginal swab contents was smeared onto a glass slide and inoculated onto heart infusion agar to monitor commensal flora. The remaining swab contents were resuspended in 1 mL of sterile GC broth, which was then quantitatively cultured on GC-VCNTS agar using the Autoplater 5000 spiral plating system (Spiral Biotech). The number of CFU recovered was determined after 48 hours incubation using the Spiral Biotech 530 Color Q Microbial Colony Cell Counter (Advanced Instruments) and the results were expressed as the number of CFU/ml of vaginal swab suspension. The limit of detection was 20 CFU. Vaginal smears were stained with a HEMA-3 stain and used to enumerate PMNs as a percentage of total cells for each mouse, as described (165). URT cultures were performed as follows. Mice were humanely euthanized at day 5

post-inoculation, and aseptically dissected to remove uteri, cutting just cranial to the cervix and caudal to the oviducts. Uterine horns were then laterally bisected to expose the endometrial surface, which was then gently scraped using a sterile size 15 scalpel to dislodge bacteria in the superficial layers of endometrial tissue. One mL of sterile saline was used to rinse the scraped tissue, and then collected and quantitatively cultured on GC agar as described above. Results were expressed as the number of CFU/ml of endometrial scrapings (limit of detection, 1.5 CFU/mL). Differences in colonization load between groups were compared using multiple T tests when comparing two groups, or a repeated-measures two-way analysis of variance (ANOVA) when comparing 3 or more groups; Bonferroni's post hoc analysis was used for both analyses. Differences in the duration of colonization were measured using a Kaplan-Meier survivorship curve and the log-rank (Mantel-Cox) test. All statistical analyses were performed using GraphPad Prism, version 7.01.

Measurement of cytokines and chemokines

Vaginal lavages were performed on days 1, 3, 5, and 7 post-bacterial inoculation by gently washing vaginal compartments with 30 µl of sterile saline. Four washes were combined from each mouse (120 µl) and centrifuged in a microfuge for 4 minutes to remove cellular debris and mucus; supernatants were stored at -80°C. The experiment was repeated once to test reproducibility and increase statistical power. Samples were analyzed using the Cytokine & Chemokine 26-Plex Mouse ProcartaPlex™ Panel 1 (Thermo Fisher Scientific). Arrays were analyzed using BioPlex 200 bead reader (Bio-Rad), and then background-corrected median fluorescence intensity was compared to standard curves to calculate pg/ml concentrations of each protein using BioPlex Manager

software (Bio-Rad). Results were analyzed by 2-way ANOVA, using 1/2 of the lower limit of detection as negative values. To determine the correlation between CFU and cytokine levels, *N. gonorrhoeae* vaginal swab cultures collected on days 1, 3, 5, and 7 were matched with the cytokine measurements from the same day and the same mouse. Analysis of cytokine correlation with vaginal CFU was performed by calculating the Spearman correlation coefficient.

Flow cytometry/T cell responses

Local T cell responses were assessed by intracellular cytokine staining and flow cytometric analysis of the iliac lymph nodes that drain the reproductive tract, as follows. Iliac lymph nodes were aseptically removed from euthanized mice and immediately placed in RPMI 1640 + 1% Pen/Step + 10% FBS (RPMI-CM) on ice. Single-cell suspensions were prepared by physical disruption of the tissue and filtration through 70 uM cell strainers. Live cells were counted using Trypan Blue staining and a hemocytometer. Cells were diluted to 1×10^6 cells/ml and placed in culture for 24 hours with RPMI-CM, and either stimulated (via immobilized anti-CD3 [clone 145-211C] and anti-CD28 [clone 37.51] in solution) or unstimulated. Cells were incubated with 1x BD GolgiPlug during the last 3 hours of stimulation to block protein transport and to aid accumulation of intracellular cytokines. Non-specific antibody binding was blocked by incubation with Rat Anti-Mouse CD16/CD32, clone 2.4G2 (Mouse BD Fc Block™). Cell surface staining was performed for 45 minutes on ice. Cells were fixed and permeabilized using BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (for intracellular staining of IL-17, IFN-γ, and IL-4), or eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (for intracellular staining of FOXP3) according to kit directions. Intracellular staining

was performed for 30 minutes on ice. All cells were stained for CD3 (antibody clone 145-2C11; eFluor450 conjugate) and CD4 (antibody clone RM4-5; APC-eFluor780 conjugate), as well as for either CD25 (antibody clone PC61-5; SuperBright702 conjugate) and FOXP3 (antibody clone FJK-16s; AlexaFluor488 conjugate); IL-4 (antibody clone 11B11; AlexaFluor488 conjugate), and IFN-γ (antibody clone XMG1.2; PE/Dazzle conjugate); or IL-17 (antibody clone eBio17B7, R-Phycoerythrin [PE] conjugate). Additionally, all cells were stained prior to fixation with eBioscience™ Fixable Viability Dye eFluor™ 455UV. Cells were analyzed using the BD LSR II flow cytometer. Gating and analysis were performed using FlowJo (version 10.6.2).

TGF-β depletion

Experiments involving depletion of TGF-β were performed by administering a monoclonal antibody to TGF-β (clone 1D11.16.8; BioXCell) via intraperitoneal injection, beginning one day prior to inoculation, with subsequent treatments on day 0, 2, 4, 6, and 8, as described (112).

RESULTS

Gonococcal colonization load and duration of infection in the LRT in vaginally and transcervically inoculated hTf-supplemented mice is similar

We previously reported that administration of hTf supports a significantly higher vaginal bacterial load compared to mice not given hTf (155). We also demonstrated that direct transcervical inoculation of *N. gonorrhoeae* into the uterus of hTf-supplemented mice results in vaginal as well as uterine colonization. Here we tested whether the level of vaginal colonization was different in mice inoculated vaginally versus transcervically. In two independent experiments, we introduced 10^6 CFU vaginally or transcervically into

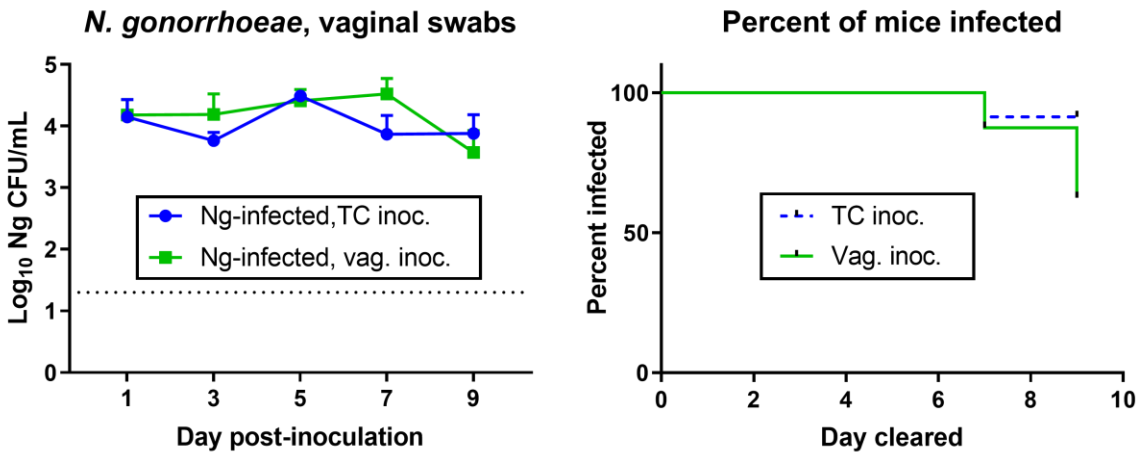

Figure 4. Vaginal bioburden and duration of infection in hTf-supplemented mice do not differ with respect to route of bacterial inoculation
(A) Mean (± SEM) CFU of *N. gonorrhoeae* recovered from vaginal swabs of mice inoculated vaginally (green) or transcervically (blue). (B) Percentage of mice infected. No statistically significant difference in colonization or duration of infection was detected between vaginally-inoculated and transcervically-inoculated mice. Data represent 15-17 mice per group from 2 combined independent experiments.

groups of hTf- supplemented mice and compared the number of CFU recovered from vaginal swabs. We found that mice in both groups had similar levels of vaginal colonization (Fig. 4A) and duration of recovery (Fig. 4B) regardless of the route of inoculation. We conclude that *N. gonorrhoeae* in the upper tract seed the LRT, that the route of inoculation does not affect vaginal colonization, and that subsequent experiments designed to compare the route of inoculation on host immune responses can be conducted without being confounded by differences in lower tract bacterial burden.

Average concentrations of pro-inflammatory cytokines and chemokines were similar in vaginally- and transcervically-inoculated mice on day 1 post-inoculation

Localized immune responses in the reproductive tract can be characterized by measuring cytokines and chemokines in vaginal lavages from experimentally infected mice (147, 210) and women with gonorrhea (70, 115). By measuring levels of local cytokines, we can glean important information about what immune cell types are present and/or being summoned to the site of infection. In addition, by comparing the bacterial burden to the levels of various cytokines, we can determine whether particular components of the immune response are sensitive to the mere presence of *N. gonorrhoeae*, or whether the response is only present at higher levels of infection. This, in turn, may be important for future studies on individual aspects of the immune response in isolation.

In order to determine the cytokine signature for *N. gonorrhoeae* URT infection in the hTf-supplemented mouse model, we collected vaginal lavages from vaginally- and transcervically-infected mice, as well as mock-inoculated and uninoculated controls that were otherwise treated identically, on days 1, 3, 5, and 7 post-inoculation. To address the concern that the physical process of inserting a catheter through the cervix might cause

inflammation due to physical damage to cervical or endometrial tissue, or by introducing vaginal bacteria to the uterus, we included two groups of uninfected control mice, one of which received mock transcervical inoculation of sterile endotoxin-free saline and the other remained uninoculated. Several pro-inflammatory cytokines (GM-CSF, MIP-2, TNF-α, RANTES, and IL-6) were significantly increased compared to the uninfected control mice at 24 hours post-inoculation in *N. gonorrhoeae*-inoculated mice regardless of route of inoculation (Fig. 5). Notably, mock-inoculated mice (Fig 5, black bars) had nearly identical levels of all cytokines to uninoculated mice (Fig. 5, white bars) throughout the time-course, even at day 1, indicating that little to no inflammation was caused by transcervical inoculation. By day 3 post-inoculation, most of the cytokines that were elevated in bacterially inoculated animals on day 1 had decreased, with only the average concentrations of TNF-α, RANTES, and MIP-2 still significantly elevated. MIP-1α and MIP-1β were significantly elevated on days 1 and 3 in vaginally-inoculated mice (green bars), but not in transcervically-inoculated mice (blue bars).

Cytokine/chemokine levels are strongly correlated with LRT bioburden in vaginally-inoculated mice but not transcervically-inoculated mice

Next, we sought to determine whether the observed inflammatory response was dependent on the level of bacterial colonization. For this analysis, we plotted the bacterial burden as determined by vaginal swab cultures against the corresponding cytokine levels on the X and Y axes, respectively, for each individual mouse at each time point at which both data parameters were determined (days 1, 3, 5, and 7). We then calculated the Spearman correlation coefficient for each cytokine measured. An interesting pattern emerged from this analysis. Most cytokines that were significantly elevated in infected mice were significantly correlated with vaginal bacterial burden in vaginally-inoculated,

Cytokine[a]	Function	Significantly elevated		Correlated with LRT CFU	
		Vaginal	TC[b]	Vaginal	TC
GM-CSF	Granulocyte and monocyte proliferation, recruitment	+	+	–	–
Gro-α		+	–	–	+
IL-1β	Pro-inflammatory, many functions	+	+	+	–
IL-6		+	+	–	–
TNF-α		+	+	+	+
RANTES	Activation, chemotaxis for T cells, granulocytes	+	+	+	–
MIP-1α	Recruitment, activation of neutrophils	+	+	+	–
MIP-2		+	+	+	+
MIP-1β	Recruitment of NK cells, monocytes, others	+	+	+	–
IFN-γ	Macrophage activation; pleiotropic effects	–	–	+	–
Eotaxin	Chemotaxis for eosinophils	–	–	+	–
IL-2	T cell proliferation	–	–	+	–
IL-10	Immunosuppressive pleiotropic effects	–	–	+	–
IL-17	Pro-inflammatory; Th17 associated	–	–	+	–
IL-22	Th17-associated; sometimes anti-inflammatory	–	–	+	–
IP-10	IFN-γ-induced; many effects	–	–	+	–

Table 1. Summary of cytokine elevation and correlation with bacterial burden

[a]Listed are all cytokines detected in vaginal washes. Each row indicates whether or not the cytokine was significantly elevated in comparison to uninfected controls, and whether or not it was significantly correlated with vaginal bacterial burden, for transcervically-infected mice or vaginally-infected mice.

[b]TC, transcervical inoculation

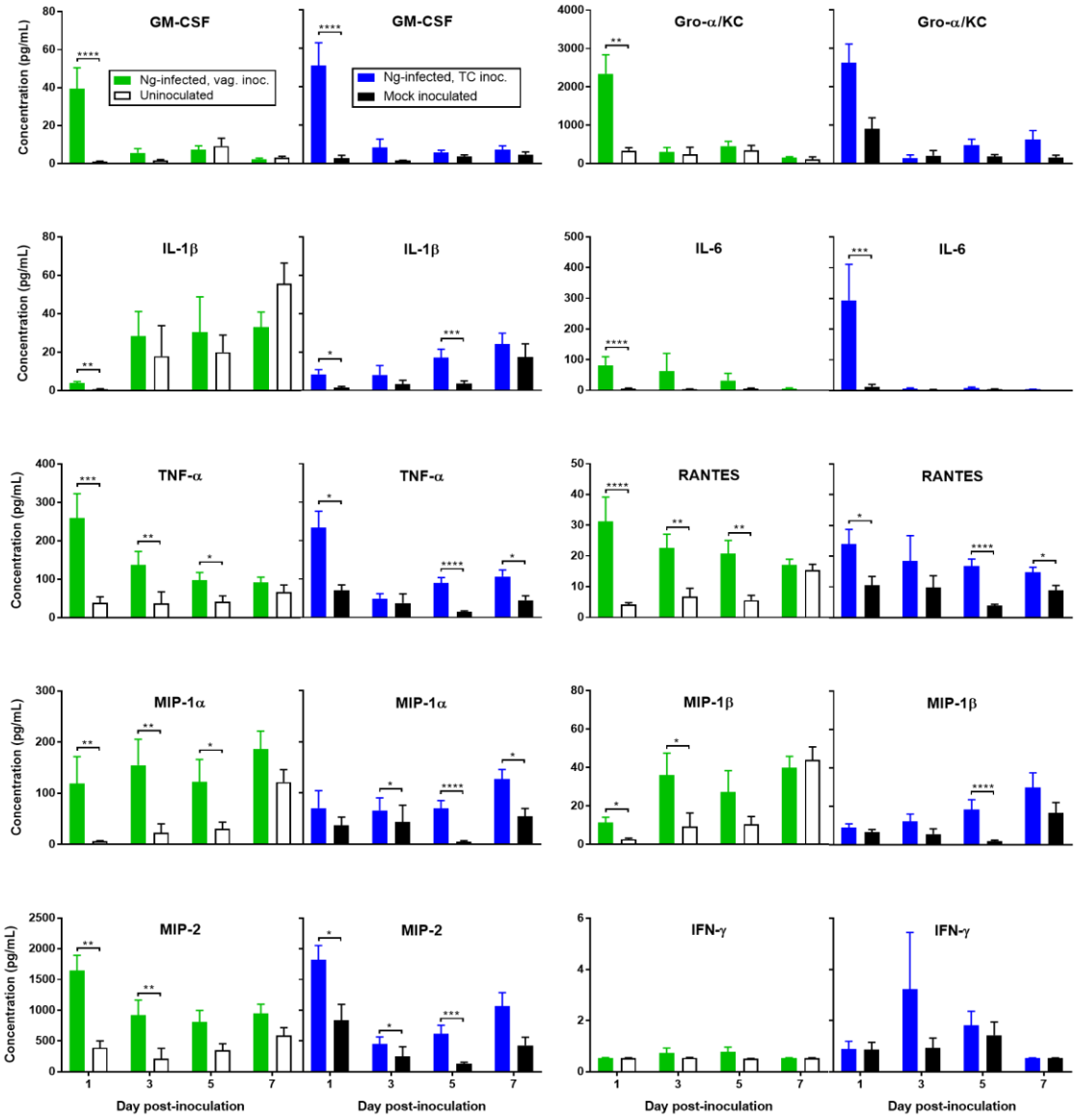

Figure 5. The average level of pro-inflammatory cytokines during infection is similar in vaginally and transcervically infected mice

Cytokines and chemokines were quantitated via multiplex immunoassay of vaginal washes collected from *N. gonorrhoeae*-infected mice (vaginally or transcervically inoculated) and uninfected controls (mock-transcervically-inoculated and uninoculated) at the indicated time points. Bars represent means +/- the standard error of the mean. Statistical significance determined using multiple unpaired T tests (one per time point) of log-transformed data and corrected for multiple comparisons using the Bonferroni-Dunn method. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. Data represent 15-17 mice per group from 2 independent experiments.

but not transcervically-inoculated mice (Table 1). Cytokines that fit this pattern include IL-1β, RANTES, MIP-1α, and MIP-1β (Fig. 6). Two cytokines, TNF-α and MIP-2, were significantly elevated and also positively correlated with vaginal bacterial burden for both routes of inoculation (Table 1). Only Gro-α levels correlated with vaginal bacterial burden for transcervically-inoculated but not vaginally-inoculated mice (Table 1). Intriguingly, due to wide variations in cytokine levels in infected mice, numerous cytokines were not significantly elevated compared to uninfected mice, but were significantly correlated with vaginal bacterial burden in vaginally- but not transcervically-inoculated mice. These include: IFN-γ, eotaxin, IL-2, IL-10, IL-17, IL-22, and IP-10.

Overall, these results suggest that some components of the inflammatory response may only be elicited at higher levels of infection, including the cytokines listed above that were not significantly elevated but were positively correlated with bacterial burden. Furthermore, the area of the reproductive tract exposed to bacteria may also impact the immune response to infection, since we see different patterns in the response to transcervical inoculation and vaginal inoculation.

The number of *N. gonorrhoeae* in the LRT is strongly correlated with a lower number and percentage of Th1 and Th2 cells

The predominant response to LRT infection in mice is a Th17 response, which leads to the recruitment of PMNs and inflammatory effectors (53). Evidence of an *N. gonorrhoeae*-induced Th17 response was detected in humans with gonorrhea (56) and is consistent with infections due to other pyogenic cocci. In contrast, Th1 and Th2 responses are suppressed through TGF-β-dependent mechanisms, which result in a poor

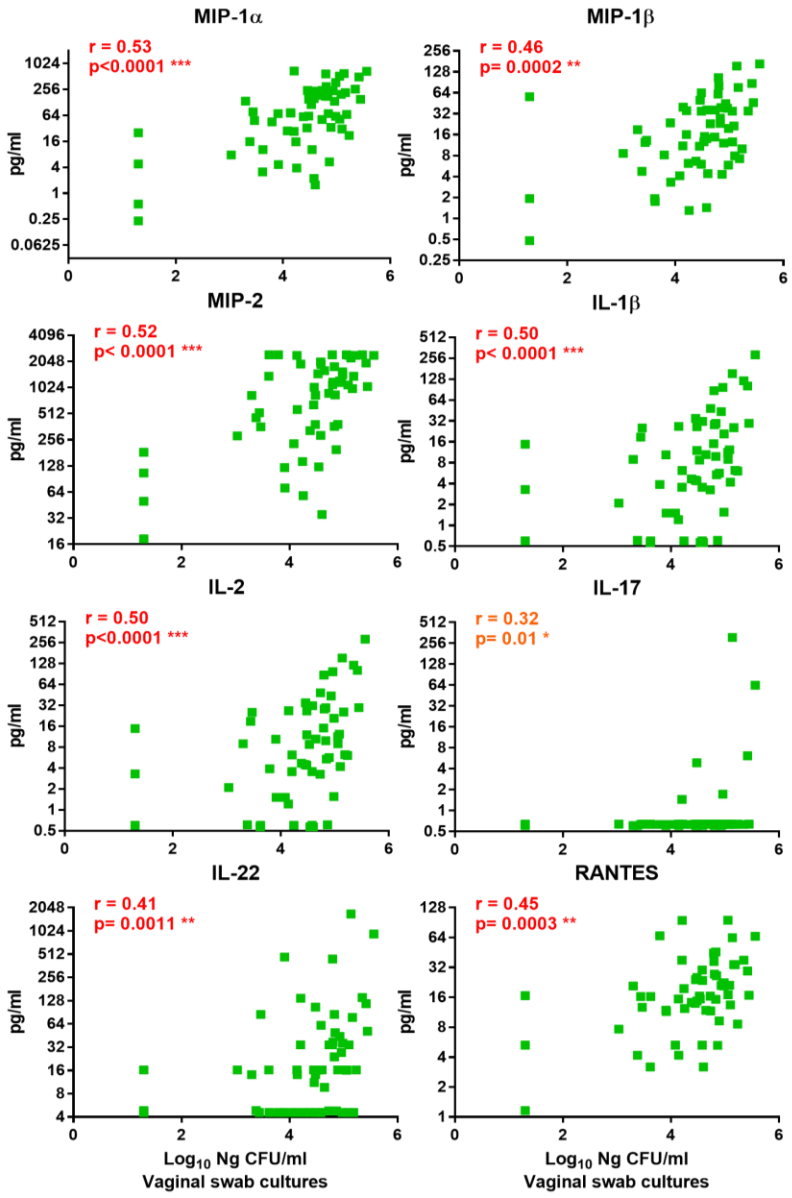

Figure 6. Correlation between vaginal bacterial burden and concentrations of selected cytokines in vaginally-inoculated hTf-supplemented mice
Each point represents the concentration of the indicated cytokine (y-axis) and the corresponding vaginal bacterial burden (x-axis) throughout the experiment. Each panel represents combined data from four time points (1, 3, 5, and 7 days post-inoculation) of two independent experiments (n = 15–17). MIP1α, MIP-1β, IL-1β and RANTES showed a significant correlation with bioburden in vaginally inoculated mice only; MIP-2 also showed a correlation with bioburden in transcervically inoculated mice (Table 1). Levels of IL-2, IL-17 and IL-22 showed this correlation in vaginally inoculated mice, but the average concentrations of these cytokines was not elevated over that of uninfected control mice. The r-values represent the Spearman correlation coefficient. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

humoral response and the lack of memory responses (107). To our knowledge, there are no published studies on the T cell response to *N. gonorrhoeae* URT infection in women. Thus, we sought to explore the T cell response to URT infection in the hTf-supplemented mouse model by analyzing subsets of CD4 T cells obtained from groups of mice that were inoculated with *N. gonorrhoeae* vaginally or transcervically. An uninfected control group that was mock-inoculated transcervically with sterile saline was tested in parallel. Five days after inoculation, we collected iliac lymph nodes from mice and quantitively cultured the URT for *N. gonorrhoeae*.

For the following analyses, we grouped mice in two different ways: first, in groups defined by inoculation route, to determine the effect of vaginal or transcervical inoculation, and second, in groups defined by whether mice had positive LRT and URT cultures or only positive LRT cultures, in order to determine whether the site of infection had an effect. Vaginally and transcervically mock-inoculated mice were used as controls. Mice with negative vaginal cultures collected on the 4 days prior to URT culture were excluded.

To determine whether infection led to any significant differences in the overall number of T cells, which could affect other analyses, we quantified CD4 T cells, and found that the average number of T cells was similar in all groups, regardless of whether infected mice were grouped by route of inoculation (Fig. 7A) or by the type of infection (Fig. 7B). However, when we examined the number of T cells with respect to the average bacterial burden in the LRT for individual mice (Fig. 7C), we found a negative correlation between increased LRT bacterial burden and numbers of CD4 T cells; no such correlation was evident for URT bacterial burden (Fig. 7D).

We next analyzed the numbers and percentages of Th1, Th2, Th17, and regulatory T cells. No cell types were significantly increased or decreased in number (data not shown) or as a percentage of the total number of CD4 T cells in infected mice compared to uninfected controls, regardless of whether mice were grouped by inoculation route (Fig. 8A) or by the site of infection (Fig. 8B). However, when we examined whether T cell subsets might correlate with the LRT and URT bacterial burden in individual mice, a different story emerged. Increased LRT bacterial burden was strongly correlated with a decrease in Th1 and Th2 cells, both as a percentage of total CD4 cells (Fig. 9A) and in number (Fig. 10A). No such correlation with respect to Th1 and Th2 cells was evident with the URT bacterial burden. Instead, increased URT bacterial burden correlated with a decreased percentage of regulatory T cells (Fig 9B), and increased numbers of Th17 cells (Fig 10B).

TGF-β depletion results in significant clearance of infection in vaginally-inoculated but not transcervically-inoculated mice

The negative correlation between Th1 and Th2 cells and LRT bacterial burden was strongly reminiscent of several previously published studies that showed a decrease in Th1 and Th2 cells associated with LRT infection (172). Gonococcal suppression of the Th1 and Th2 response was shown to be dependent upon transforming growth factor β (TGF-β), and depletion of TGF-β abrogated this immunosuppression, allowing a greater humoral response and accelerated clearance of *N. gonorrhoeae* (112). This work was conducted using the LRT infection model developed by our laboratory, and thus the impact of hTf supplementation and URT infection on the effect has not yet been explored. Therefore, we next chose to examine the effect of depleting TGF-β in the context of hTf supplementation to determine whether TGF-β depletion would affect

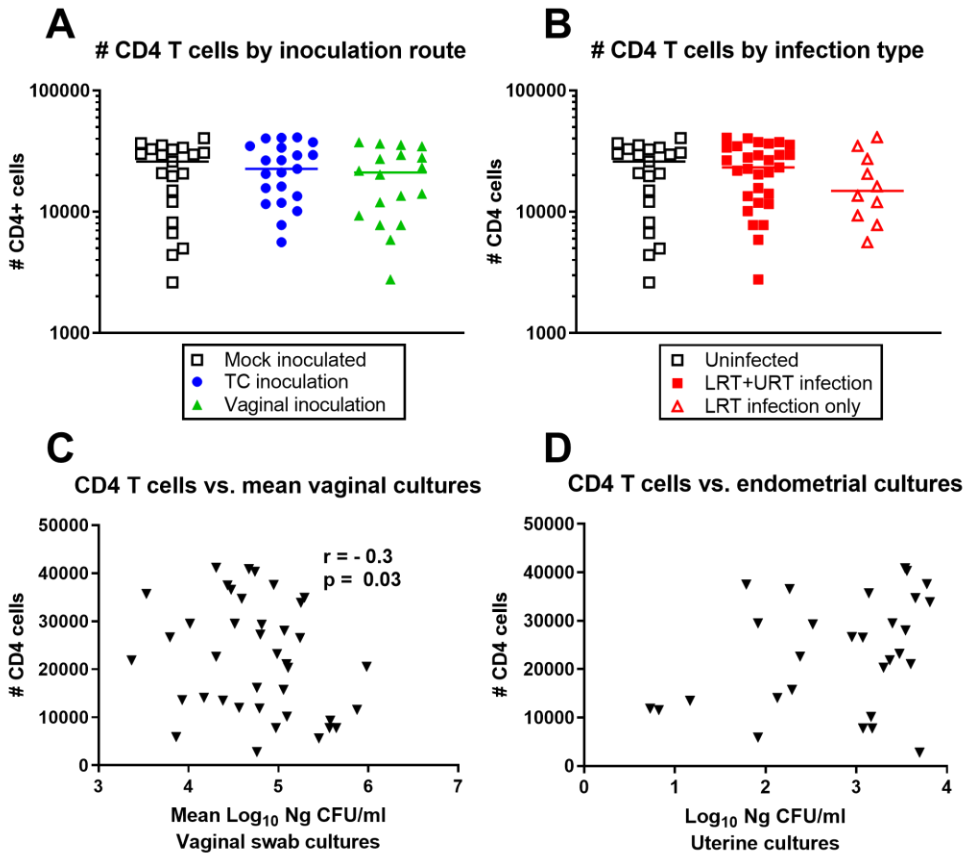

Figure 7. Numbers of CD4 T cells were negatively correlated with vaginal bacterial burden in infected mice but not significantly different from uninfected mice A and B: Number of iliac lymph node CD4 T cells in uninfected mice (white squares), compared to mice categorized by (A) the route of inoculation or (B) site of infection. C and D: Correlation of CD4 T cell numbers with bacterial burden. Each point represents the number of CD4 T cells (y-axis) and the corresponding (C) vaginal CFU averaged over four days of infection (x-axis) or (D) the endometrial CFU recovered on day 5 from each individual mouse. Expression of surface markers was analyzed by flow cytometry in mice sacrificed 5 days post-inoculation. Statistical analysis: one-way analysis of variance with Dunnett's multiple comparisons test (A and B) or Spearman's correlation (C and D). Data represent four independent experiments, total n = 20-22 mice per group. R-values represent the Spearman correlation coefficient.

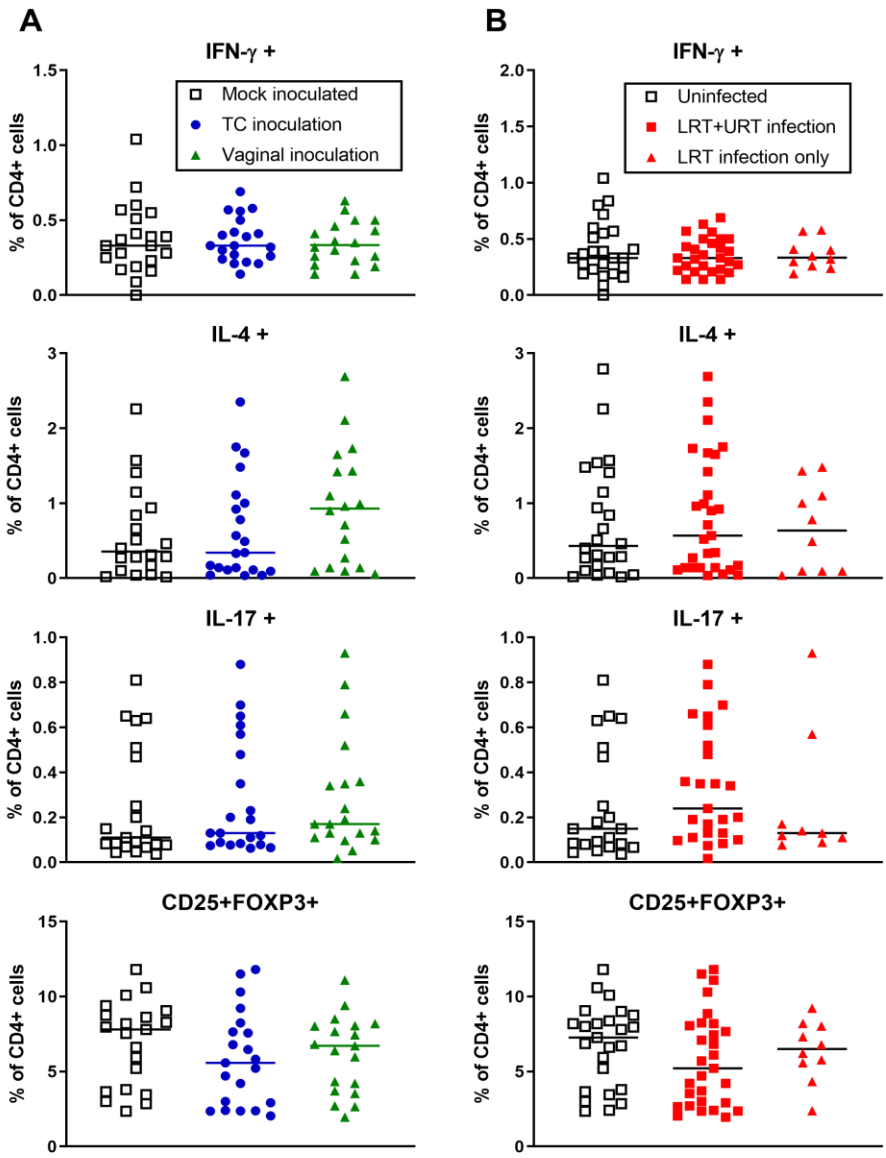

Figure 8. CD4 T cell subsets were not different when mice were grouped by route of inoculation or site of infection

The percentage of iliac lymph node CD4 T cells expressing IFNγ (Th1), IL-4 (Th2), IL-17 (Th17), or CD25 and FOXP3 (Treg) was determined in mice inoculated with *N. gonorrhoeae* vaginally or transcervically and in uninfected mice. No differences were found between mice grouped by (A) route of inoculation or (B) the site of infection. (A) T cell subsets in uninfected (white squares), transcervically inoculated (blue circles) or vaginally-inoculated (green triangles) mice. (B) T cell subsets in uninfected mice (white squares), mice with LRT and URT infection (red squares), or LRT infection alone (red triangles). Statistical analysis: one-way analysis of variance with Dunnett's multiple comparisons test. Data represent four independent experiments, total n = 20-22 mice per group.

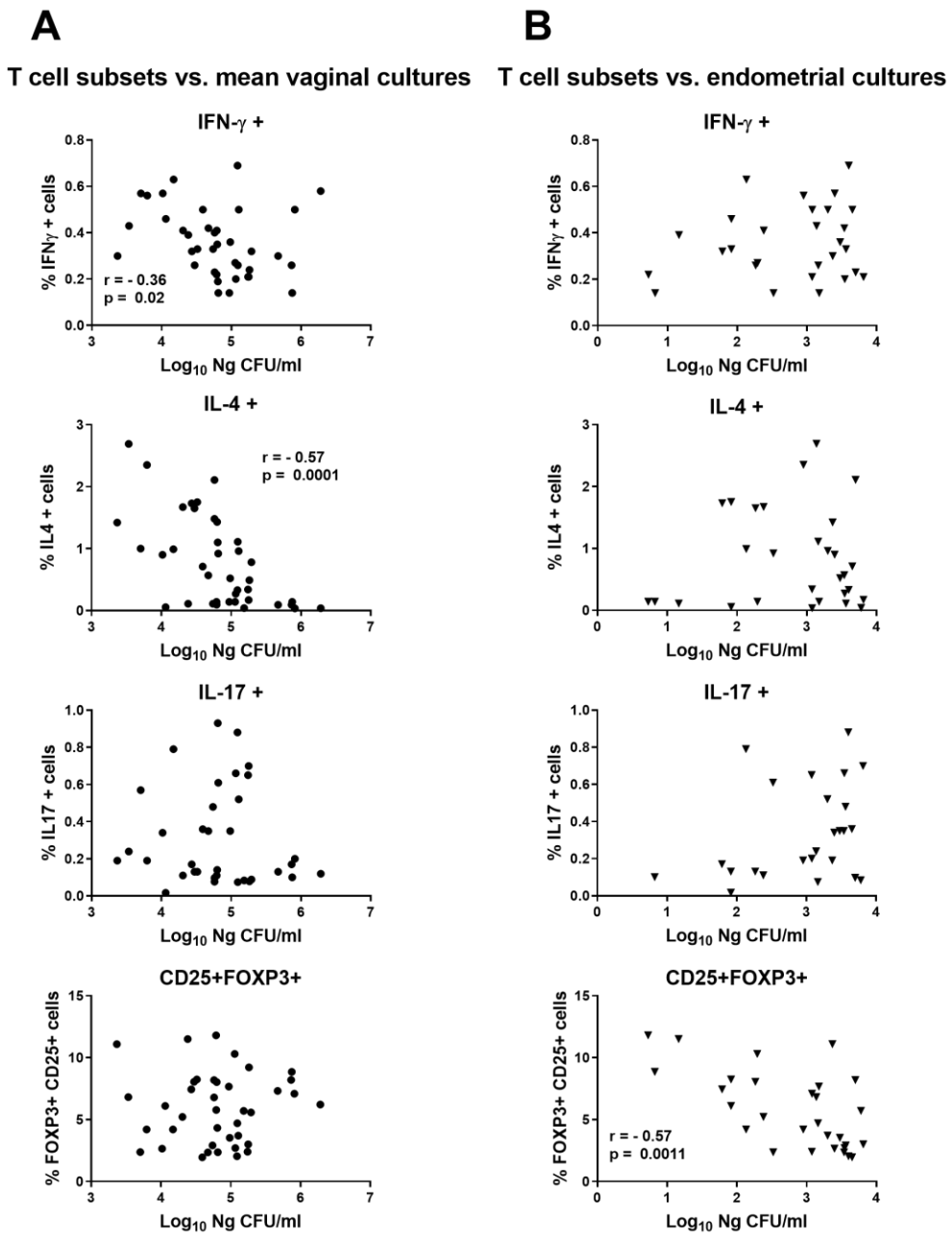

Figure 9. The percent of Th1 and Th2 cells is negatively correlated with vaginal bacterial burden while the percent of regulatory T cells is negatively correlated with endometrial bacterial burden

Each point represents the percent of the indicated T cell subset in iliac lymph nodes (y-axis) and (A) the vaginal CFU averaged over four days of infection (x-axis) or (B) the corresponding endometrial CFU recovered on day 5 (x-axis) from each individual mouse. Expression of surface and intracellular markers was analyzed by flow cytometry in mice euthanized 5 days post-inoculation. The r-values represent the Spearman correlation coefficient.

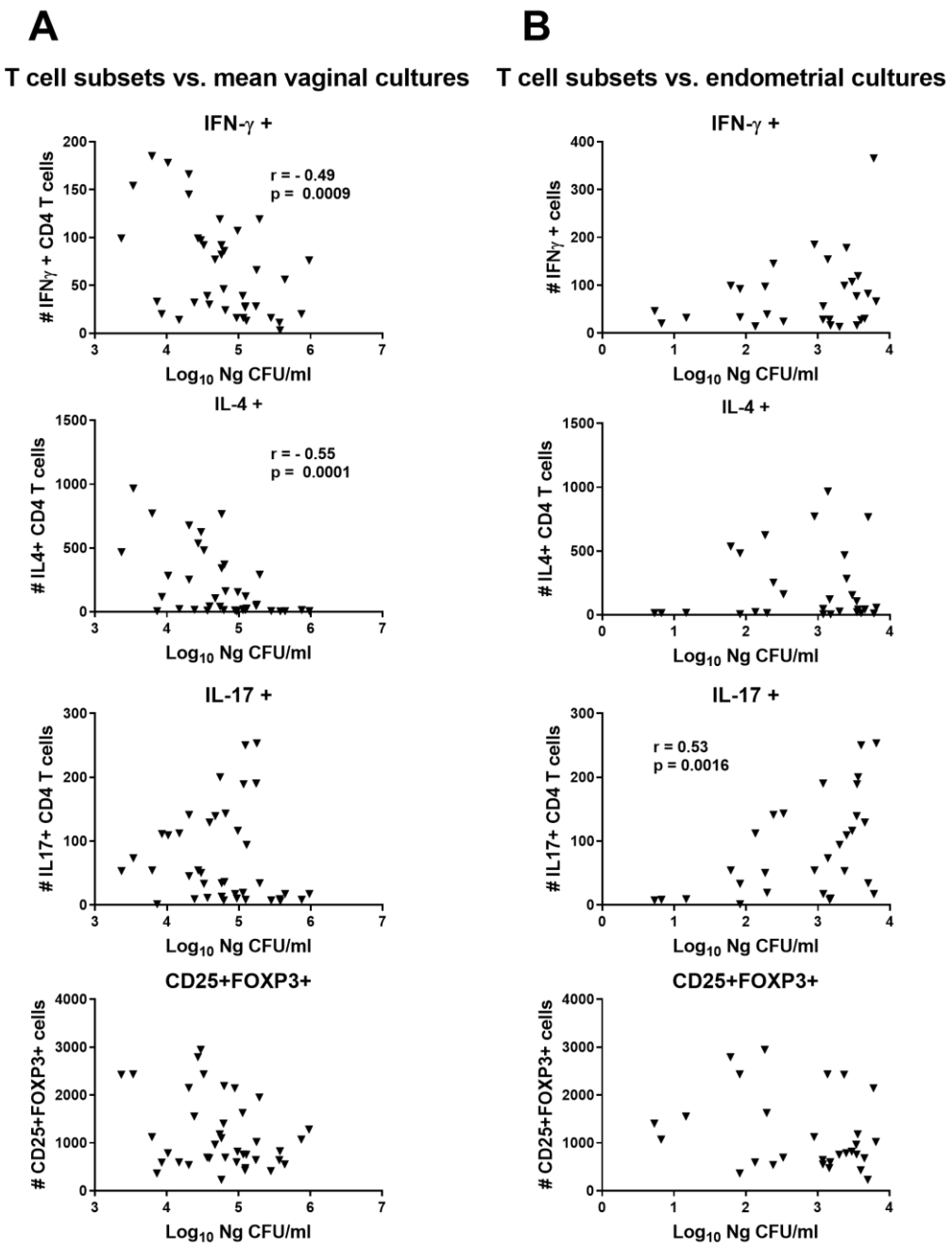

Figure 10. The number of Th1 and Th2 cells is negatively correlated with vaginal bacterial burden while the number of Th17 cells is positively correlated with endometrial bacterial burden

Each point represents the number of the indicated T cell subset in iliac lymph nodes (y-axis) and (A) the vaginal CFU averaged over four days of infection (x-axis) or (B) the corresponding endometrial CFU recovered on day 5 (x-axis) from each individual mouse. Expression of surface and intracellular markers was analyzed by flow cytometry in mice euthanized 5 days post-inoculation. The r-values represent the Spearman correlation coefficient.

clearance of *N. gonorrhoeae* in hTf supplemented mice, which have a higher bioburden compared to mice not given hTf. In addition, since the decrease in Th1 and Th2 cells that we observed was correlated with vaginal but not endometrial bacterial burden, we further wished to test whether the effect of TGF-β depletion was affected by the route of inoculation. We hypothesized that TGF-β depletion would facilitate clearance of *N. gonorrhoeae* infection, but that the effectiveness would decrease with hTf supplementation. In order to test this hypothesis, we used 6 different groups of mice. Four groups of mice were vaginally inoculated with *N. gonorrhoeae*. Of these, one group was supplemented with hTf and treated with an antibody to deplete TGF-β; two other groups received anti-TGF-β alone or hTf alone, and the fourth group was not given either treatment. Two additional groups were transcervically inoculated with *N. gonorrhoeae*, one of which was treated with both hTf and anti-TGF-β, and the other only with hTf. All mice received estradiol and antibiotics as per our standard protocol. Vaginal colonization of all mice was monitored via vaginal swabs on days 1, 3, 5, 7, and 9 post-inoculation.

Vaginally inoculated mice displayed distinctly different levels of colonization between treatment groups (Fig. 11). As reported (155), mice that received hTf without anti-TGF-β had a markedly higher colonization load than mice that were not given hTf or anti-TGF-β (Fig. 11A, red and blue lines, respectively). No difference in the bioburden was found in unsupplemented mice given anti-TGF-β versus no treatment (Fig. 11A, green and blue lines, respectively). However, consistent with the previous report (112), unsupplemented mice given anti-TGF-β had a significantly accelerated clearance rate compared to unsupplemented, untreated mice (Fig. 11B, green and blue lines, respectively).

TGF-β depletion had a similar effect on vaginally-inoculated hTf-supplemented mice, although the effect, while significant, was not as pronounced. Mice that were hTf-supplemented and TGF-β depleted were colonized at a lower level compared to hTf-supplemented mice, with a significant difference observed at day 9 post-inoculation (Fig. 11A, brown and red lines, respectively). When the percent of infected mice at each time point was compared, a significant difference in the clearance rate was observed, with 44% of TGF-β-depleted hTf-supplemented mice infected on day 9 versus 87% of hTf-supplemented mice infected at this time point (Fig 11B, brown and red lines, respectively).

In contrast, transcervically inoculated mice remained colonized at approximately equal levels regardless of whether they were treated with hTf alone or hTf in combination with anti-TGF-β. Sixty-two and 69% of mice were colonized, respectively, at day 9 post-inoculation; there was no significant difference in either bacterial burden or clearance rate between the two groups (Fig. 11C and 11D).

We conclude that hTf supplementation reduces but does not eliminate the effect of TGF-β depletion in vaginally inoculated mice. Further, the immune response incurred by TGF-β depletion is either stronger or more effective in vaginally-inoculated mice compared to transcervical inoculation. These results suggest one of two possible interpretations. The first possibility is that the presence of *N. gonorrhoeae* in the URT early in infection in transcervically inoculated mice alters the immune response so that a protective response does not develop. The second possibility is that the immune response is the same to both types of infection regardless of the initial site of infection, but is less effective at clearing infections that are present in the URT.

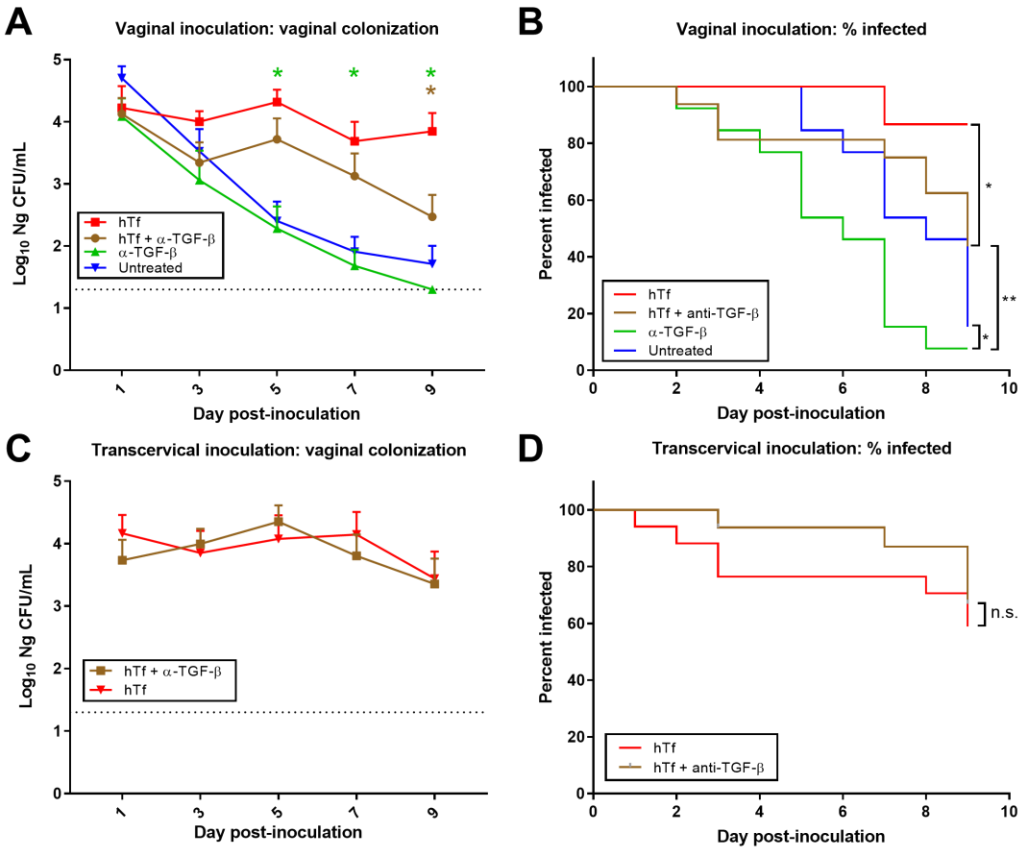

Figure 11. TGF-β depletion accelerates clearance of infection in vaginally-inoculated but not transcervically-inoculated mice

Groups of female BALB/c mice were treated with anti-TGF-β antibody, either alone (green) or in conjunction with hTf supplementation (brown). Mice that were given hTf alone (red) or left untreated (blue) were tested in parallel. Mice were vaginally inoculated (A, B) or transcervically inoculated (C, D). Left panels (A, C) show recovery of *N. gonorrhoeae* from vaginal swabs (mean ± SEM); right panels (B, D) indicate the percentage of mice infected. Green asterisks indicate a statistically significant difference between mice treated with anti-TGF-β alone versus those given both anti-TGF-β and hTf; brown asterisk indicates a statistically significant difference between mice given both anti-TGF-β and hTf and those given hTf alone. *, $p < 0.05$; **, $p < 0.01$. Statistical significance determined by repeated measures two-way analysis of variance with Bonferroni's correction for multiple comparisons (A, C) or log-rank (Mantel-Cox) test (B, D).

DISCUSSION

In this work, we have further characterized a newly developed mouse model of *N. gonorrhoeae* URT infection for the purpose of examining questions related to immune responses to gonococcal PID. In this model, mice are given hTf to provide a usable iron source in the URT and to better mimic human infection. Here we examined the proinflammatory response to infection and investigated CD4 T cell responses, with a focus on Th17 and the possible suppression of Th1 and Th2 responses, which have been characterized by others in the murine LRT infection model (107). Our results demonstrate that the cytokine/chemokine response to *N. gonorrhoeae* infection in this model is dependent on bacterial burden and location of bacteria in the reproductive tract, with inoculation by the vaginal route more stimulatory than direct inoculation of *N. gonorrhoeae* into the uterus. We also showed that immunosuppression of Th1 and Th2 responses is correlated with the LRT bacterial burden but does not appear to be affected by route of inoculation or URT bacterial burden. In contrast, the URT bacterial burden is negatively correlated with regulatory T cells. Further, depletion of TGF-β, previously shown to relieve Th1/Th2 immunosuppression and accelerate infection clearance in the LRT infection model, had the expected effect on infection in vaginally- but not transcervically-inoculated mice, suggesting that there may be fundamental differences in immune stimulation and/or protection in different regions of the reproductive tract. The impact that the site of initial infection had on host responses raises several intriguing immunological questions. This finding is a caveat to the use of transcervical inoculation to establish URT infection, however, in that the ability of such models to replicate the immune response to URT infection may be confounded by bypassing factors that dictate immunological events during natural ascension.

Data on the cytokine response to *N. gonorrhoeae* URT infection are sparse. Islam (85) and Francis (55) published studies in which they examined the expression of various cytokines after transcervical inoculation of *N. gonorrhoeae* in mice in different stages of the estrous cycle or given estradiol or progesterone. These investigators found that the response to infection was strongly dependent on the estrous stage at the time of bacterial inoculation, with progesterone-dominant mice showing a much stronger response as defined by increases in IL-1α, IL-1β, MIP-1α, and MCP-1 at 6 hours post-inoculation. However, in that model, all pro-inflammatory cytokines were restored to pre-inoculation levels by 18 hours post-inoculation in estrus-stage mice, while the response was sustained or even increased in diestrus-stage mice (85). These results contrast with ours in that we see sustained elevation of proinflammatory cytokines for up to 7 days post-inoculation, although the response is most pronounced in the first 24 hours.

Studies utilizing the fallopian tube organ culture model have also demonstrated induction of TNF-α upon incubation with *N. gonorrhoeae,* which is partially responsible for a reduction of ciliated cell activity and sloughing of cells (104, 117). This report is consistent with our results, in which *N. gonorrhoeae*-infected mice, regardless of inoculation route, exhibit a strong increase in TNF-α that is strongly correlated with bacterial burden. It is unclear what proportion of TNF-α induction is due to bacterial presence in the URT, but the fact that the response is similar at day 1 post-inoculation in both vaginally- and transcervically-inoculated mice, and that it correlates with LRT bacterial burden in both groups suggests that TNF-α is induced primarily by interaction with cells in the LRT. Future studies should examine the contribution of the increase of

TNF-α to pathology in the oviducts, as TNF-α has been demonstrated to be a major contributor to oviduct pathology in models of chlamydial PID (131).

Although we have demonstrated the induction of proinflammatory cytokines in this model that are consistent with data from the fallopian tube organ culture model of *N. gonorrhoeae* URT infection, one outstanding question remains to be answered regarding the extent to which the hTf supplementation mouse model recreates human PID, which is whether tubal pathology results from URT infection. Future studies utilizing immunohistochemical staining of URT tissue will help to answer this question. One difficulty in performing such studies is the choice of time points at which to euthanize animals and collect tissue. In our studies, we see peak induction of pro-inflammatory cytokines at 24 hours post-inoculation; however, the URT bacterial burden peaks 5 days after inoculation. In order to gain an accurate picture of when damage occurs in relation to infection ascension, an extended time course will likely be required, in which tissue is collected from infected animals over at least 7 days post-inoculation. Further, since repeated episodes of PID are associated with increased rates of infertility, ectopic pregnancy, and chronic pain, future histopathology studies should also examine the effect of repeated infections in mice.

This work represents the first effort, to our knowledge, to define the local inflammatory response in relation to the *N. gonorrhoeae* bacterial burden of individual animals. By combining correlation data with data on group means, we gained insight into the varying contributions of different anatomical locations in the reproductive tract in eliciting different types of cytokines. This matter is complicated by the fact that the

majority of vaginally-inoculated mice will have ascending infection by day 5 post-inoculation and we have no data to show when ascension occurs.

Our discovery that the T cell response is strongly correlated with bacterial burden is also novel, but is consistent with previous literature, particularly our finding that decreased numbers and percentages of Th1 and Th2 cells correlate with increased bacterial burden. There are two possible explanations for this result. One is that the presence of more *N. gonorrhoeae* in the LRT is somehow actively reducing Th1/Th2 cell proliferation, recruitment, or differentiation, by either direct or indirect means. However, it is also possible that a natural variation in Th1 and Th2 cells in mice may be present, so that in mice with lower numbers of Th1 and/or Th2 cells, uncontrolled bacterial growth and survival may occur, thus creating the same correlation.

Our experiments utilizing TGF-β depletion were an attempt to determine whether artificially diverting the T cell response toward a Th1/Th2 dominant response would effectively clear infection, as previously demonstrated (112). The fact that TGF-β depletion only results in infection clearance for mice that are vaginally-inoculated suggests that either (1) the presence of *N. gonorrhoeae* in the URT prevents the development of a protective Th1/Th2 response; or (2) Th1/Th2 responses are ineffective at clearing *N. gonorrhoeae* infections that are already in the URT. Future studies, possibly utilizing other methods of inducing Th1/Th2 responses, such as administering microencapsulated IL-12, will determine which explanation is correct.

The use of *in vivo* imaging techniques to identifying ascending infection in live animals will be greatly beneficial for future uses of the hTf-supplemented mouse model. This strategy would allow us to conduct studies on the immune response in individual

animals and determine what inflammatory processes are precipitated by ascending infection. Furthermore, studies involving the artificial induction of immune responses, such as those described in this work utilizing TGF-β depletion, would then be able to determine whether particular types of host response can prevent ascending infection. The latter will be especially important for vaccine studies, as prevention of PID is a primary goal for STI vaccines, and an animal model in which to validate protection in that context would be invaluable.

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CHAPTER 3: A female mouse model of *Neisseria gonorrhoeae/Chlamydia muridarum* upper reproductive tract coinfection

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The work presented in this chapter is the sole work of Claire Costenoble-Caherty with the following exceptions: W. Zhu and A. Macintyre performed flow cytometry staining and analysis for T cell quantification, and A. Macintyre conducted cytokine/chemokine measurements.

ABSTRACT

Pelvic inflammatory disease is a serious complication of *Neisseria gonorrhoeae* (*N. gonorrhoeae*) and *Chlamydia trachomatis* (*C. trachomatis*) infections. Concurrent infections by these pathogens are difficult to study due to the lack of an upper reproductive tract coinfection model. Here we describe a model that uses 17β-estradiol-treated mice and human transferrin to support gonococcal endometrial infection, and transcervical inoculation of the mouse pathogen *Chlamydia muridarum* (*C. muridarum*) to allow chlamydial infection in estrogenized mice. Simultaneous inoculation of both pathogens resulted in endometrial infection in 80-90% of mice for at least 10 days. Vaginal proinflammatory cytokines were significantly elevated in *N. gonorrhoeae*-infected and coinfected mice compared to *C. muridarum* alone, and the chlamydial bioburden was significantly lower in coinfected mice. Pre-infection with *N. gonorrhoeae* followed by *C. muridarum* was also successful and did not show differences in colonization by either pathogen. We also demonstrated the *in vivo* efficacy of ceftriaxone

and doxycycline against coinfection. We conclude that while *C. muridarum* colonization is influenced by order of exposure, this model is limited for pathogenesis studies due to estradiol-induced immunosuppression, particularly on the immune response to *C. muridarum*. This model and the culturing techniques we have developed, however, should be useful for development of dually active therapeutics against Pelvic Inflammatory Disease.

INTRODUCTION

The sexually transmitted pathogens *Chlamydia trachomatis* (*C. trachomatis*) serovars D-K and *Neisseria gonorrhoeae* (*N. gonorrhoeae*) are responsible for an estimated 127 million and 87 million annual infections, respectively (140). In the United States, infections due to these pathogens are the most frequently reported notifiable infections to the Center for Disease Control and Prevention (214). Acute urogenital infections manifest similarly for both pathogens, typically as urethritis in men and cervicitis, with or without urethritis, in women. A high percentage of infections are asymptomatic, and pharyngeal and rectal infections by these pathogens are also common in both sexes (81). Gonorrhea/chlamydial coinfections are also common; as many as 42.7 − 70% of adolescents and young adults with gonorrhea have a concurrent chlamydial infection (49, 123, 142). A lower proportion (0.7% − 11%) of patients with *C. trachomatis* infections are coinfected with *N. gonorrhoeae*, likely due to the lower prevalence of gonorrhea (123, 142).

More serious disease occurs when bacteria ascend from the lower urogenital tract to the testes, epididymis, uterus or fallopian tubes (29). In women, ascending cervical

infections can result in Pelvic Inflammatory Disease (PID), which is defined as inflammation due to infection of the endometrium and fallopian tubes (12). If untreated, PID can lead to irreversible damage to the reproductive tract. Approximately 18% of women with a history of PID experience chronic pelvic pain due to scar tissue and adhesions in the upper reproductive tract (URT), and infection-mediated damage to the fallopian tubes can lead to infertility and ectopic pregnancy (12, 29). *C. trachomatis* and *N. gonorrhoeae* are the first and second most common causes of PID when a bacterial agent can be identified. *Mycoplasma genitalium* and several facultative- and obligate-anaerobic bacteria are associated with the remainder of cases (75, 135). As with cervical infections, PID due to concurrent *C. trachomatis* and *N. gonorrhoeae* infection is common, and the number of PID cases due to coinfection is often similar to the number of cases due to *N. gonorrhoeae* alone, as is consistent with the more frequent occurrence of *C. trachomatis* coinfection in individuals with gonorrhea (25, 59).

Despite similarities in disease, *C. trachomatis* and *N. gonorrhoeae* are quite different pathogens, with the chlamydiae being obligate intracellular bacteria and gonococci found mostly extracellular with an intracellular niche. It is therefore reasonable to postulate that the pathology and host responses that occur during concurrent infections differ from that which occurs during infections with either single organism. *N. gonorrhoeae* is more inflammatory, as evidenced by the greater purulence of exudates in acute infections compared to acute *C. trachomatis* infections (81). Gonorrhea can also cause more severe PID as supported by recent studies from Western Australia which showed that while chlamydial PID was more common in the cohort studied, there was a greater risk of PID and chance of hospitalization due to PID in women with gonorrhea or

N. gonorrhoeae/*C. trachomatis* coinfection compared to *C. trachomatis* infection alone (166, 167). Tissue damage due to chlamydia-induced inflammation is well documented in laboratory animal models (132) and the immune response appears to be initiated by intracellular chlamydial growth in infected epithelial cells (46, 104). Both pathogens induce tumor necrosis factor α (TNF-α), interleukin 1, and other pro-inflammatory cytokines that can mediate damage (46, 104). Host adaptive immune responses in the context of coinfection are not defined but are intriguing, as *C. trachomatis* induces transient protection against reinfection that is mediated by antibodies and interferon γ-producing CD4 T cells (64), while *N. gonorrhoeae* induces a Th17-mediated inflammatory response and a suppressed adaptive response that is characterized by reduced Th1 and Th2 cell proliferation (172).

Animal models of chlamydial and gonococcal genital tract infection have illuminated interactions between these pathogens and the female reproductive tract. Surrogate models of *C. trachomatis* human infection that utilize *Chlamydia muridarum* or *Chlamydia caviae* in their native mouse mice and guinea pig hosts, respectively, are often used to study chlamydial pathogenesis *in vivo* (48). *C. muridarum* infection of mice causes URT disease that closely mimics that caused by *C. trachomatis* infection in women (48). Oviduct pathology, which is characterized by hydrosalpinx, occurs after 5-6 weeks (48, 168). Like *C. muridarum* infection in mice, *C. caviae* infection in guinea pigs results in a self-limited infection with a duration of 3-4 weeks, during which infection of the oviducts occurs in about 80% of infected animals (44, 48). In contrast, there are no surrogate *Neisseria* species that mimic gonorrhea in humans. *N. gonorrhoeae* genital tract infection has only been established in chimpanzees and female mice; attempts to infect

other animals with *N. gonorrhoeae*, including guinea pigs, were unsuccessful (13). Mice are transiently susceptible to *N. gonorrhoeae* when in the proestrus stage of the reproductive cycle, and 17β-estradiol can be administered to prolong susceptibility (165). Gonococci are recovered from vaginal swabs for 10-14 days, depending on the estradiol formulation used, and can be detected in the vaginal lumen, associated with vaginal and cervical cells, and within the lamina propria (92). *N. gonorrhoeae* is recovered from the URT of 17-20% of mice, but the numbers of bacteria recovered are low (92). A similar finding was reported by other in which low numbers of viable gonococci were recovered from the murine endometrium for only 6 - 72 hours after transcervical inoculation (85).

Differences in the susceptible stage of the rodent estrous cycle for *N. gonorrhoeae* and *C. muridarum* infection create a technical obstacle for establishing coinfection by these pathogens. In contrast to *N. gonorrhoeae*, *C. muridarum* infection of mice is most successful in the post-ovulatory phases of the murine reproductive cycle, and pre-treatment with progesterone or multiple inoculations over three days is used to increase the rate of infection and the number of inclusion-forming units (IFU) isolated from cervicovaginal swab cultures (48). We previously reported that *N. gonorrhoeae*/*C. muridarum* coinfection could only be established in mice by first infecting mice with *C. muridarum* for 2 to 6 days before treating with 17β-estradiol followed by vaginal inoculation with *N. gonorrhoeae* (210). Both pathogens were recovered from vaginal swabs for an average of 8-9 days in a 10-day study period and chlamydial inclusions and gonococci were seen in genital tract tissue. This model mimicked reported observations on coinfection in humans, including the enhanced inflammation produced by coinfection

compared to single infections, and the increase in gonococcal bioburden in *C. muridarum*-infected mice (193, 210).

Here we sought to improve the *N. gonorrhoeae*/*C. muridarum* coinfection model by addressing two of its limitations. First, the lack of URT involvement by *N. gonorrhoeae* prevents studies on concurrent URT infections, which are the major source of morbidity and mortality associated with these pathogens. Recently, however, we demonstrated that administration of human transferrin (hTf), a host-restricted iron source for *N. gonorrhoeae*, allows establishment of gonococcal URT infection for at least 7 days (155). By adapting this new protocol for coinfections, we hypothesized we could establish URT infection with both pathogens. Second, the previously developed coinfection model (210) can only be used to study events that occur when *N. gonorrhoeae* encounters a host with a pre-existing chlamydial infection. While this scenario is likely common due to the higher prevalence of chlamydial infection and clinical evidence that *C. trachomatis* may persist in women for as long as two years (58), other orders of exposure do occur. Here, by altering the route of chlamydial inoculation, we were able to establish models of simultaneous coinfection and of primary gonococcal infection followed by a secondary chlamydial infection. We also examined the local inflammatory and cellular responses in simultaneously infected mice and provide proof of concept of the usefulness of this model for testing the *in vivo* efficacy of candidate antibiotics against concurrent URT infections by these pathogens.

MATERIALS AND METHODS

Bacterial strains and *in vitro* propagation

N. gonorrhoeae strain FA1090 [*porB1b*; serum-resistant; arginine, hypoxanthine, and uracil auxotroph], was originally isolated from a female with disseminated infection (90). Supplemented GC agar and culture conditions used to prorogate piliated FA1090 bacteria were as described (36); GC agar with antibiotic selection (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 (165). *C. muridarum* strain Nigg was propagated in L929 mouse fibroblast cells (210). The titer of inclusion-forming units (IFU) in each stock was determined using the Pathfinder *Chlamydia* Direct Fluorescence Assay kit (BioRad).

Experimental murine infection

All animal experiments were conducted according to the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care and the Uniformed Services University Institutional Animal Care and Use Committee. Female BALB/c mice [4-5 weeks, Charles River Laboratories (Wilmington, MA)] were housed with autoclaved food, water and bedding and allowed to acclimate to the animal facility for 10 days. Mice in the diestrus stage of the estrous cycle were identified by vaginal smear and treated with three doses of 0.5 mg of 17β-estradiol suspended in sesame oil, beginning two days prior to inoculation (165). Mice were also treated with streptomycin and vancomycin to reduce colonization by commensal organisms as described (165). Recombinant human transferrin (hTf) (Sigma) (8 mg/dose) was given daily by intraperitoneal (IP) injection beginning 2 hours prior to bacterial inoculation and over the course of the experiments. Bacterial inocula were prepared using *N. gonorrhoeae*

harvested from agar plates and *C. muridarum* elementary bodies purified on Percoll-gradients and stored at -80° C as described (139, 165). Three separate experiments were conducted in which mice were inoculated transcervically with 1×10^6 CFU of *N. gonorrhoeae,* 5×10^5 IFU of *C. muridarum*, a combined suspension of the same number of both organisms, or saline (mock-inoculated control) using flexible tubing from a 26-gauge intravenous catheter (Zoetis) attached to a 0.2 ml pipette tip (n = 9 − 10 mice per group). The infection protocol for experiments using this simultaneous inoculation is shown in Fig. 12. Two experiments were conducted in which mice were infected transcervically with *N. gonorrhoeae* prior to inoculation with *C. muridarum* 24 hours later; all other treatments were the same as described above.

Vaginal swabs were quantitatively cultured for *N. gonorrhoeae* and *C. muridarum* on days 1, 3, 5, and 10 post-inoculation as described (210). URT cultures were conducted on days 3, 5, and 10 post-inoculation as follows. Mice were humanely euthanized using ketamine/xylazine as per the American Veterinary Medical Association guidelines, and the URT removed by cutting anterior to cervix and posterior to oviducts. The URT was placed in a sterile petri dish and dissected longitudinally along each uterine horn, and the lumens gently scraped with a scalpel in a bath of 1 ml sterile PBS. The endometrium was rinsed with the sterile saline, which was collected for quantitative cultures for *N. gonorrhoeae* as described (210). Remaining tissue was suspended in sterile sucrose-phosphate-glucose (SPG) buffer, frozen at -80° C and later homogenized using Omni Tissue Homogenizer (Omni International, Kennesaw, GA, USA). Tissue homogenates were inoculated onto monolayers of L929 cells to quantitate viable *C. muridarum* bacteria. Culture results were expressed as CFU/ml of vaginal swab suspension (limit of

detection: 20 CFU) or of endometrial wash (limit of detection: 2 CFU) for *N. gonorrhoeae* and as IFU/ml of vaginal swab suspension or of tissue homogenate (limit of detection: 10 IFU for both body sites) for *C. muridarum*. Statistical significance of differences in colonization load was determined by a mixed-model analysis with Bonferroni's multiple comparisons test.

Cytokine and chemokine protein analysis

To assess the inflammatory response to infection, estradiol-treated, hTf-supplemented BALB/c mice were inoculated with *N. gonorrhoeae*, *C. muridarum*, or both pathogens simultaneously (7 − 8 mice/group) as described above. A fourth group was inoculated with saline to provide a mock-inoculated control group for comparison. Vaginal lavages were performed on days 2 and 5 post-bacterial inoculation by gently washing vaginal compartments with 30 µl of sterile saline. Four washes were combined from each mouse (120 µl) and centrifuged in a microfuge for 4 minutes to remove cellular debris and mucus; supernatants were stored at -80°C. The experiment was repeated once to test reproducibility and increase statistical power. Samples were analyzed using a 20-Plex magnetic bead panel (LMC0006M; Invitrogen) to determine levels of cytokines/chemokines. Arrays were analyzed using BioPlex 200 bead reader (Bio-Rad), and then background-corrected median fluorescence intensity was compared to standard curves to calculate pg/ml concentrations of each protein using BioPlex Manager software (Bio-Rad). Statistical significance was calculated using a repeated measures two-way analysis of variance, with Bonferroni's multiple comparisons test.

Intracellular cytokine staining

To examine the CD4 T cell response to infection, BALB/c mice were inoculated with either single pathogens, both pathogens simultaneously, or uninfected, (n = 4 − 5 mice per group per time point) and the iliac lymph nodes were aseptically removed from groups of mice at day 10 or day 20 post-inoculation. Single-cell suspensions were prepared and left unstimulated or stimulated overnight with 50 ng/ml PMA (Sigma-Aldrich Corp.) plus 750ng/ml ionomycin (Sigma-Aldrich Corp.). During the last 8 hours of culture, GolgiSTOP (BD Biosciences) was added to block cytokine secretion. Following stimulation, cells were stained for viability (Live/Dead Aqua, Invitrogen) and surface labelled with CD3-BV421 (clone 145-2C11, Biolegend), CD4-APC-Cy7 (clone GK1.5, BD Biosciences) and CD8-FITC (clone 53-6.7, BD Biosciences). Cells were then permeabilized using CytoFix/Perm (BD Biosciences). After washing, canonical Th1, Th2, and Th17 cytokines were labelled using anti-IL-2-PcP-Cy5.5 (JES6-5H4, Biolegend), anti-IFN-γ-APC (clone XMG1.2, Biolegend), anti-IL-4- BV711 (clone 11B1, Biolegend) and anti-IL-17-PE (clone TC11-18H10, BD Biosciences). Cells were fixed in 1% paraformaldehyde (Sigma) in PBS prior to acquisition on an LSRII flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Treestar). The experiment was repeated one time for a total n = 8-10 mice per group per time point, and statistical significance was determined using a one-way ANOVA.

Antibiotic treatment of coinfection

BALB/c mice were infected with *N. gonorrhoeae* and *C. muridarum* as shown in Fig. 12. Five days after bacterial inoculation, mice were randomized into two experimental groups (n = 8 mice/group). One group received a single IP injection of

ceftriaxone (15 mg/kg), and doxycycline (10 mg/kg) and four additional daily doses of doxycycline at 24-hr intervals (36, 39). The second group was untreated. Vaginal swabs were cultured for *N. gonorrhoeae* and *C. muridarum* 2 days prior to treatment, 2 hours before treatment, and on days 3 and 5 post-treatment. Endometrial tissues were cultured on day 5 post-treatment as described above.

RESULTS

Estradiol-treated mice can be infected with *C. muridarum* when inoculated transcervically

A major challenge to developing a murine gonococcal/chlamydial coinfection model is that mice are susceptible to these pathogens at different stages of the reproductive cycle. Based on the demonstration that *C. muridarum* ascends to the URT about 7 days after vaginal inoculation (27), we first hypothesized that that URT coinfection might be possible if we inoculated mice vaginally with *C. muridarum* and delayed 17-β estradiol treatment for 2-5 days followed by hTf treatment and transcervical inoculation with *N. gonorrhoeae*. However, URT cultures taken on days 3, 5 and 10 post-*N. gonorrhoeae* inoculation (n = 3 mice/time point) yielded *C. muridarum* from only 2 of 9 mice. The percentage of *N. gonorrhoeae*-positive cultures was also low (Table 2).

Others have shown that sustained *C. trachomatis* infection of mice can be established in mice without progesterone treatment if the bacteria are inoculated transcervically rather than vaginally (48, 64). We therefore tested whether *C. muridarum* might successfully establish URT infection in estradiol-treated mice if inoculated transcervically. In a pilot experiment, *C. muridarum* was recovered from the URT of 75% of mice on day 3 post-bacterial challenge (Table 2), and we therefore next tried

transcervical inoculation of estradiol-treated, hTf-treated mice with both pathogens suspended in a single inoculum, using the experimental design outlined in Figure 12. The

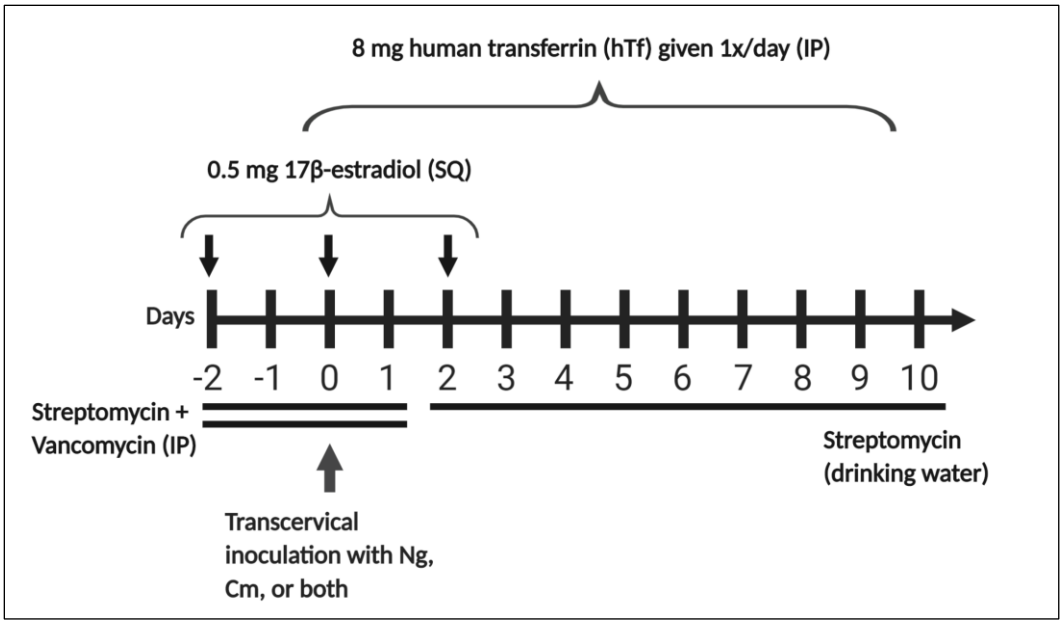

Figure 12. Protocol for establishing URT coinfection using simultaneous transcervical inoculation of each pathogen.

Mice in the diestrus stage of the estrus cycle were treated subcutaneously every other day with 0.5 mg of estradiol sodium benzoate suspended in oil for a total of three doses starting two days prior to bacterial inoculation (D-2). hTf (8 mg/mouse) was administered by intraperitoneal injection on the day of bacterial inoculation (D0) and for the next 8 consecutive days. Antibiotics (streptomycin and vancomycin) were administered on days -2 through day 9 to suppress the overgrowth of commensal flora that occurs with estradiol treatment (92). Mice were inoculated transcervically on day 0 with *N. gonorrhoeae* alone, *C. muridarum* alone, a mixture of both organisms, or mock-inoculated with sterile saline.

first iteration of this experiment showed that 33% had positive *C. muridarum* endometrial cultures and 100% of mice had positive *N. gonorrhoeae* endometrial cultures (Table 2). Subsequent repeat experiments showed that 67% and 59% of co-inoculated mice had positive endometrial cultures for *N. gonorrhoeae* and *C. muridarum*, respectively, on days 3, 5 or 10 post-inoculation (Fig. 13). We conclude that the use of transcervical inoculation allows *C. muridarum* to by-pass estrogen-related restrictions in the LRT.

N. gonorrhoeae **significantly reduced the** ***C. muridarum*** **bacterial burden early in infection of the LRT and URT in mice that were simultaneously inoculated with both pathogens**

As the first step towards characterizing the simultaneous coinfection model, we sought to determine whether the duration of infection or colonization load of either organism was significantly altered by concurrent infection with the other organism. Mice were inoculated with *N. gonorrhoeae*, *C. muridarum* or both pathogens, and the vaginal burden of each pathogen was measured on days 1, 3, 5, and 10 post-inoculation. URT colonization was analyzed by sacrificing 3 − 4 mice per group on days 3, 5, and 10 post-inoculation, and culturing endometrial washes for *N. gonorrhoeae* and tissue homogenates for *C. muridarum*. Combined data from three independent experiments are shown in Fig. 13. Vaginal cultures showed sustained infection with both organisms for 10 days, with ~10^3 − 10^5 CFU of *N. gonorrhoeae* and ~10^2 − 10^4 IFU of *C. muridarum* recovered per ml of vaginal swab suspension, and a slight increase in the number of *C. muridarum* IFU over time (Fig. 13A and 13B). Gonococcal colonization load was unaffected by chlamydial coinfection (Fig. 13A). In contrast, the *C. muridarum* bioburden in the LRT was significantly lower in coinfected mice compared to mice

Order of inoculation	*C. muridarum* inoculation route	% of coinfected mice with positive URT cultures[a] (# positive/total #)	
		C. muridarum	*N. gonorrhoeae*
***C. muridarum* first**	Vaginal	22 (2/9)	33 (3/9)
***C. muridarum* only**	Transcervical	75 (3/4) [b]	N/A
***N. gonorrhoeae* first**	Transcervical	55 (5/9)	55 (5/9)
Simultaneous	Transcervical	33 (3/9)	100 (9/9)

Table 2. Summary of experiments using different routes and orders of inoculation with *N. gonorrhoeae* and *C. muridarum* exposure

[a,] Percent infected based on number of mice that had a positive URT culture on day 3, 5, 7 or 10.

[b,] For this experiment, mice received estradiol and no hTf

infected with *C. muridarum* alone (Fig. 13B) ($p = 0.005$). The difference between the two groups became more pronounced as infection progressed, with the greatest difference evident at days 5 and 10 post-inoculation, at which point *C. muridarum* IFUs were approximately 5 − 10-fold lower in mice co-inoculated with *C. muridarum* and *N. gonorrhoeae* (Fig. 13B). As infection progressed to day 10, the number of chlamydial IFUs recovered from coinfected mice appeared to increase (Fig. 13B), suggesting that factors related to *N. gonorrhoeae* infection challenge *C. muridarum* during this time period.

We observed a similar colonization pattern in the URT. Fewer chlamydial IFU were recovered from co-inoculated mice compared to mice inoculated only with *C. muridarum* (Fig. 13F) and only 50 − 60% of coinfected mice were culture-positive for *C. muridarum* on days 3 and 5 of infection compared to 100% of mice inoculated only with *C. muridarum* (Fig. 13D). By day 10 post-inoculation, *C. muridarum* colonization in the URT was similar in both groups, with 90% of co-inoculated mice culture positive, compared to 100% of mice inoculated with *C. muridarum*-only (Fig. 13D). As in the LRT, *N. gonorrhoeae* infection seemed unaffected by chlamydial coinfection, as the percentage of mice that were culture-positive for *N. gonorrhoeae* (Fig. 13C) and the number of CFU recovered (Fig. 13E) were similar between coinfected and *N. gonorrhoeae*-only mice at all time points. We conclude that the simultaneous infection protocol reproducibly establishes URT coinfection in a high percentage of mice, but that chlamydial colonization is negatively impacted by concurrent infection with *N. gonorrhoeae*.

Pre-infection with *N. gonorrhoeae* followed by *C. muridarum* inoculation did not result in a reduced chlamydial bioburden as seen in simultaneously inoculated mice

Acquisition of a second sexually transmitted pathogen can also occur in individuals who are already infected with a different sexually transmitted pathogen. In this case, pathology and/or the induction of host responses by the initial infection may alter the outcome of the secondary infection (181). To determine whether coinfection with *C. muridarum* could be established in *N. gonorrhoeae*-infected mice, we inoculated estradiol-treated, hTf-treated mice with *N. gonorrhoeae* and introduced *C. muridarum* transcervically 24 hours later. As observed in the simultaneous inoculation model, the number of mice with positive *N. gonorrhoeae* cultures was lower on days 3 and 5 compared to day 10 (Fig. 14). *C. muridarum* colonization in the URT was similar for both orders of inoculation and ranged from 57 − 83%, depending on the time-point and did not show evidence of reduced colonization by *C. muridarum* in mice with a concurrent *N. gonorrhoeae* infection.

Vaginal proinflammatory cytokine and chemokine levels were significantly higher in mice inoculated with *N. gonorrhoeae* alone or both pathogens compared to *C. muridarum* alone

Due to the simplicity of inoculating mice with both bacteria at the same time, we chose to further characterize the simultaneous infection model. We first compared the localized vaginal cytokine response on days 2 and 5 post-inoculation in mice inoculated with *N. gonorrhoeae*, *C. muridarum*, or both pathogens. We detected significantly elevated levels of IL-1α, TNF-α, and MIP-1α in mice inoculated with *N. gonorrhoeae* only compared to mice inoculated with *C. muridarum* but not to mock-inoculated control mice. Co-inoculated mice had increased IL-1β and IFN-γ compared to mice inoculated with *C. muridarum* or *N. gonorrhoeae* alone and elevated MCP-1 compared to *C.*

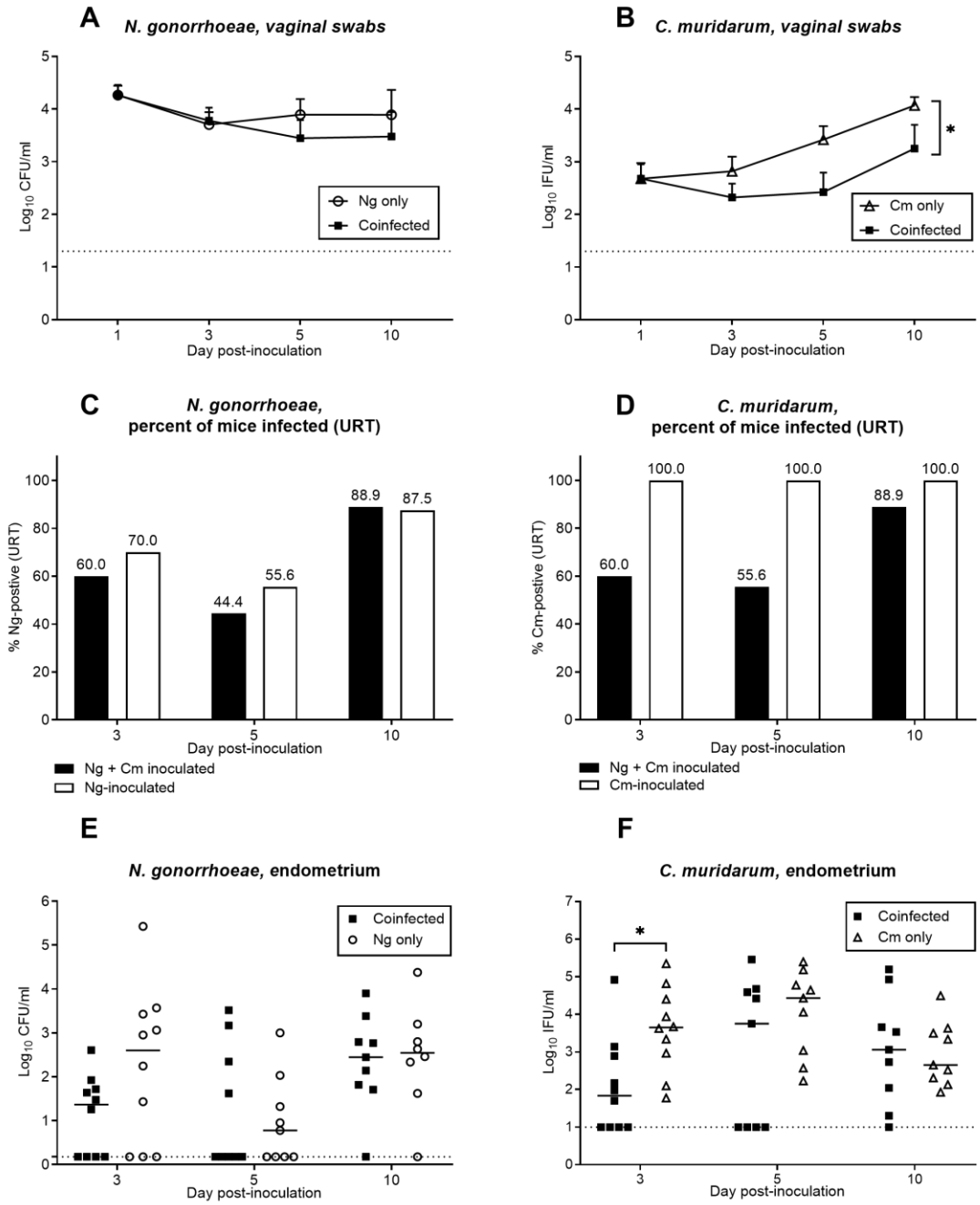

Figure 13. Simultaneous inoculation results in sustained infection of lower and upper genital tracts and lower recovery of *C. muridarum* in coinfected mice
(A and B) Mean ± standard error of the mean (SEM) gonococcal CFU (A) or chlamydial IFU (B) recovered per ml of vaginal swab suspension. Open symbols represent mice inoculated with a single pathogen; solid symbols represent co-inoculated mice. The *N. gonorrhoeae* vaginal colonization load was not affected by the presence of *C. muridarum*. In contrast, recovery of *C. muridarum* from vaginal swabs in coinfected mice was significantly lower than

from mice inoculated with *C. muridarum* alone ($p = 0.005$). (C and D) Percent of mice with positive URT cultures for (C) *N. gonorrhoeae* and (D) *C. muridarum* at each time point. (E and F) Bioburden in the URT expressed as (E) number of *N. gonorrhoeae* CFU recovered per ml of endometrial washes (F) number of *C. muridarum* IFU per ml of endometrial tissue homogenate. *N. gonorrhoeae* endometrial colonization was not different in coinfected mice compared to mice infected with *N. gonorrhoeae* alone. *C. muridarum* colonization in the URT was significantly lower on day 3 in mice concurrently infected with *N. gonorrhoeae* compared to mice infected with *C. muridarum* alone ($p = 0.033$). Horizontal bars indicate the median. The results shown in panels A and B are from three combined experiments with a total of 28 mice per group at day 1 and 8 − 10 mice per group at day 10 (decreasing numbers due to sacrificing mice for URT cultures). Results shown for URT cultures in panels C, D, E, and F were obtained from 3 − 4 mice in each experiment at each time point. The limit of detection (dotted lines) was used for negative cultures. Statistical significance of LRT and URT culture data was determined using a mixed model analysis, with Bonferroni's multiple comparisons test.

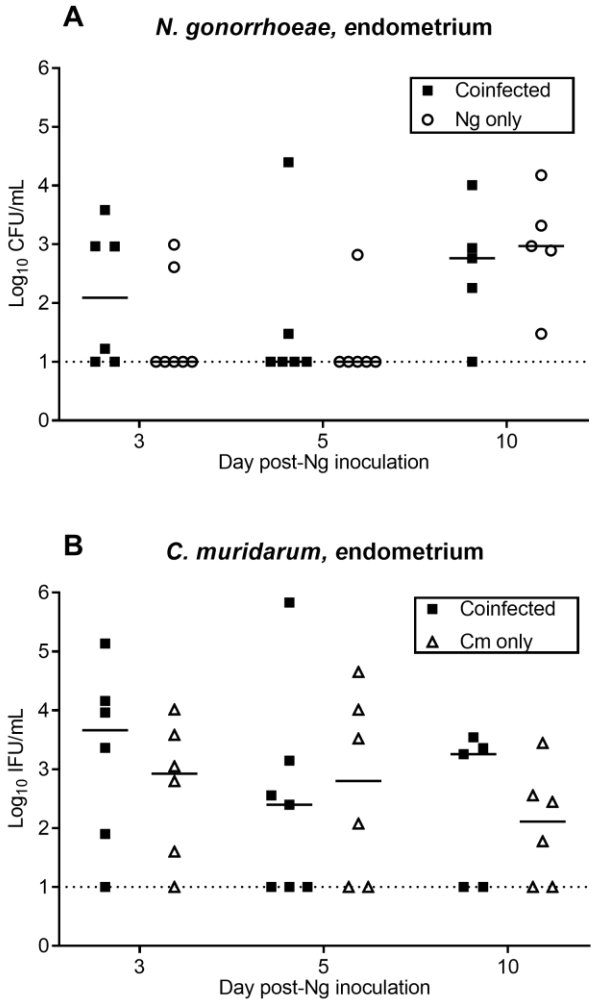

Figure 14. No difference was observed in recovery of either pathogen from the URT of mice inoculated with *N. gonorrhoeae* first compared to mice inoculated with either single pathogen

(A) Number of gonococcal CFU recovered per ml of endometrial washes (B) Number of chlamydial IFU per ml of endometrial tissue homogenate. Solid symbols denote mice inoculated with both organisms; open symbols denote animals inoculated with either single pathogen. Similar to the LRT model, lower percentages of mice were culture-positive for *N. gonorrhoeae* on days 3 [50% (3/6) and 28.5% (2/7) of mice] and day 5 [16.5% (1/6) and 33% (2/6)] in the *N. gonorrhoeae*-only and co-inoculated groups, respectively, compared to 80-100% of mice on day 10. Horizontal bars represent the median; results are from two independent experiments with a total of 5-6 mice sacrificed per group per time point.

muridarum-only and mock-inoculated mice (Fig. 15). Surprisingly neither TNF-α nor IL-6 were significantly elevated in *C. muridarum*-only mice at either time point, which was unexpected based on previous studies showing their increase in *C. muridarum* infections at similar time points (44, 159). We observed a trend towards an increased percentage of PMNs on day 5 post-inoculation in *N. gonorrhoeae*-inoculated and co-inoculated mice that was not significant compared to mock-infected controls or *C. muridarum*-inoculated mice (Fig. 16). We concluded that while a proinflammatory response was induced in *N. gonorrhoeae*-inoculated and co-inoculated mice compared to mice inoculated with only *C. muridarum*, this response was weak based on the lack of a difference compared to the PBS control group, and that the inflammatory response to *C. muridarum* was severely suppressed in this model, most likely due to the estradiol treatment.

An insignificant trend towards reduced Th1 and Th2 $CD4^+$ T lymphocyte populations was detected in mice inoculated with *N. gonorrhoeae* and both pathogens

Cellular immunity is an important component of the immune response to both *N. gonorrhoeae* and *C. muridarum/C. trachomatis*. For both pathogens, symptomatic infection is characterized by a Th17 response, while protective immunity consists of a Th1 response (60, 89, 107, 129). Thus, we sought to characterize T cell responses in the coinfection model to determine whether concurrent infections induce a different T cell response compared to single-pathogen infections. To address this question, we quantified the Th1, Th2, and Th17 cell populations within iliac lymph nodes at days 10 and 20 following inoculation with *N. gonorrhoeae*, *C. muridarum*, both pathogens, or PBS (Fig. 17). We did not observe any significant differences between experimental groups at

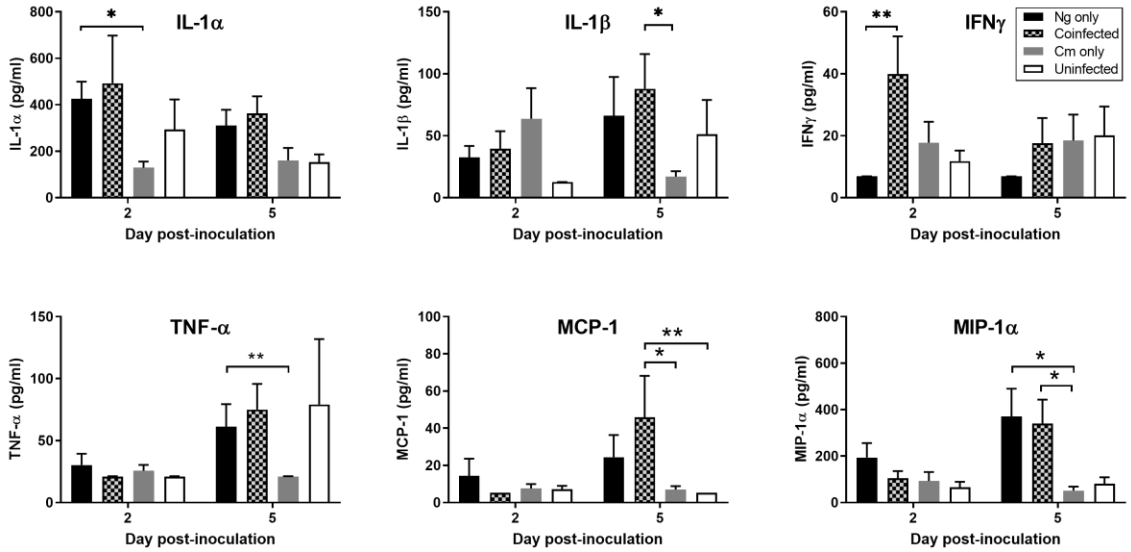

Figure 15. Significantly higher levels of vaginal proinflammatory cytokines were detected in mice inoculated with *N. gonorrhoeae*-only and with *N. gonorrhoeae* and *C. muridarum* compared to *C. muridarum* alone

Vaginal lavages were performed on mice that received either or both pathogens, or PBS (uninfected controls) (n = 14 − 15 mice per group; two experiments combined) on days 2 and 5 post-inoculation. Cytokines were quantified using a magnetic bead multiplex array. Shown are the mean cytokine concentrations in vaginal wash samples from mice with *N. gonorrhoeae* alone (black), *N. gonorrhoeae* + *C. muridarum* (patterned), *C. muridarum* alone (grey), and mock inoculated mice (white); error bars indicate the standard error of the mean. Statistical significance was calculated using a repeated measures two-way analysis of variance, with Bonferroni's multiple comparisons test. *N. gonorrhoeae*-only mice showed significant increases in IL-1α, TNF-α, and MIP-1α, while coinfected mice had increased IL-1β, IFN-γ, and MCP-1 compared to mice inoculated with only *C. muridarum*.

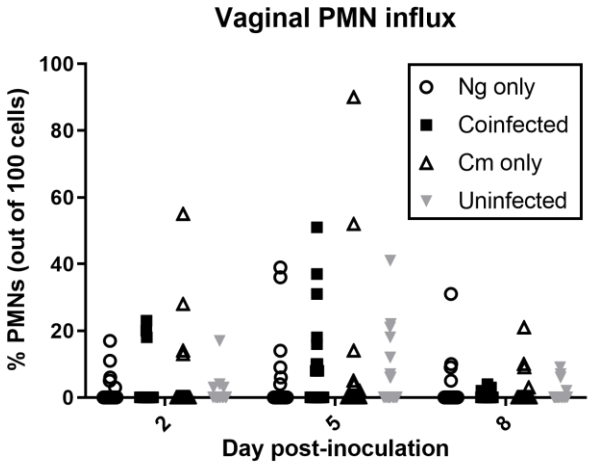

Figure 16. No difference was observed in the vaginal PMN influx between any experimental group

Vaginal swabs were collected at the indicated time points from mice inoculated with *N. gonorrhoeae* alone (open circles), *N. gonorrhoeae* + *C. muridarum* (filled squares), *C. muridarum* alone (open triangles), and mock inoculated mice (grey inverted triangles). The number of PMNs in each swab was enumerated relative to the total number of cells. Shown are the percent of PMNs for individual mice in each group (n = 25 mice per group from 3 combined experiments). Statistical significance was calculated using a repeated measures two-way analysis of variance, with Bonferroni's multiple comparisons test; no differences between groups were statistically significant.

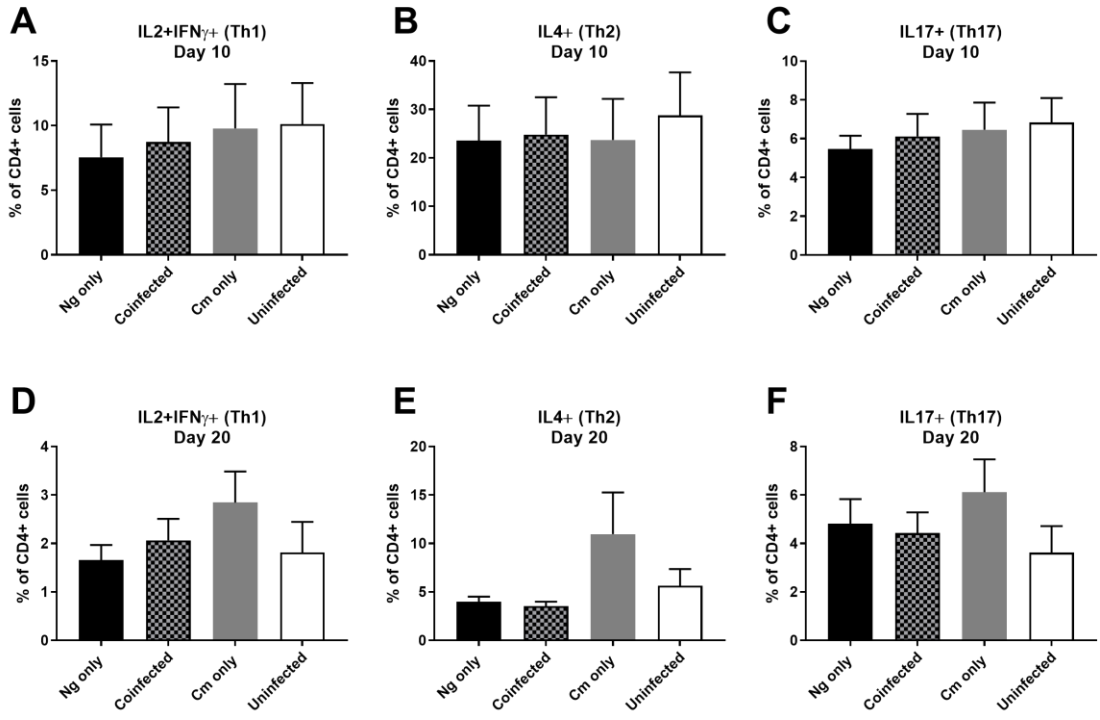

Figure 17. A trend towards reduced Th2 T cells was detected in *N. gonorrhoeae*-infected and coinfected mice

Cells from the iliac lymph nodes of mice infected with *N. gonorrhoeae* alone (black), *C. muridarum* alone (grey), *N. gonorrhoeae* + *C. muridarum* (patterned), and mock inoculated mice (white) were collected at days 10 (top row) and 20 (bottom row) post-inoculation. Cells were stimulated with PMA and ionomycin to induce cytokine production and then stained for surface CD8, CD4, CD3 and intracellular Th1, Th2 and Th17 canonical cytokines. Results depicted are combined from 2 independent experiments with 8-10 mice per group in total. The mean percent of the indicated CD4 T cell subsets is shown; error bars indicate the standard error of the mean. Statistical significance was calculated using the Kruskal-Wallis test with multiple comparisons.

either time-point. At day 20 post-inoculation, we detected a non-significant trend towards increased Th1 and Th2 CD4 cell populations *C. muridarum*-infected mice relative to the PBS control, which was not present in mice infected with *N. gonorrhoeae* alone or *N. gonorrhoeae* and *C. muridarum*. Contrary to our expectations, we did not observe a Th17 response in any infection group, except for a non-significant increase in Th17 cells in *C. muridarum*-only infected mice at day 20 post-inoculation.

A dual regimen of ceftriaxone and doxycycline significantly cleared *N. gonorrhoeae*/*C. muridarum* URT coinfection within 5 days post-treatment initiation

New antibiotics that are efficacious against gonorrhea are urgently needed (214, 229), and dually active antibiotics against *C. trachomatis* are desirable, due to the high prevalence of coinfections (6). To provide proof-of-concept for the usefulness of the URT coinfection model in testing new antibiotics, we determined whether co-administration of ceftriaxone and doxycycline, the currently recommended treatment for gonococcal and chlamydial infections (229), would clear URT coinfection. Mice were inoculated with *N. gonorrhoeae* and *C. muridarum* as described in Fig. 12, and 5 days later treated with a single parenteral dose of ceftriaxone (15 mg/kg) and the first of 5 daily doses of doxycycline (10 mg/kg) or left untreated. Vaginal swab cultures conducted on days 3 and 5 post-bacterial inoculation showed a 100% infection rate for both pathogens. *C. muridarum* and *N. gonorrhoeae* were not recovered by vaginal culture in 100% and 80% of mice, respectively on day 3 post-treatment ($p < 0.0001$); by day 5, 90% of mice were culture-negative for *N. gonorrhoeae* ($p = 0.0018$) (Fig. 18A and Fig. 18B). URT cultures conducted on day 5 post-treatment (10 days post-bacterial inoculation) showed complete clearance of *C. muridarum* and *N. gonorrhoeae* ($p = 0.011$) from the

URT (Fig. 18C and 18D). We conclude that these antibiotics are useful controls for testing the *in vivo* efficacy of antibiotics against coinfection; more detailed dose responses are needed to identify the minimum doses that will result in complete clearance of both pathogens.

DISCUSSION

Several factors complicate PID research, including variability in the criteria used to diagnose PID, the variety of microorganisms associated with PID and the high proportion of asymptomatic cases (229). *N. gonorrhoeae/C. trachomatis* coinfections are common, yet little is known about the pathogenesis and immune responses to coinfection, due in part to the lack of animal models (229)(229)(229). Here we describe the first animal model of *N. gonorrhoeae/C. muridarum* URT coinfection for use as a tool for basic and translational research. The strengths of this model include the ability to establish robust infection with both pathogens, with infection lasting for at least 10 days in 80% of animals. The use of mice as a model allows for large-scale experiments, and the ability to take advantage of the variety of immunological and genetic tools that are available for working with mice. Additionally, the fact that both gonococcal and chlamydial infections have been well-characterized in mice means that there is a strong foundation of knowledge on which to base future studies.

Limitations of this model stem from the necessary use of estradiol to maintain *N. gonorrhoeae* infection in mice. Administration of estradiol prevents the use of vaginal inoculation to establish *C. muridarum* infection. Additionally, the immunosuppressive and anti-inflammatory effects of estradiol (179, 186) seem to be particularly antagonistic

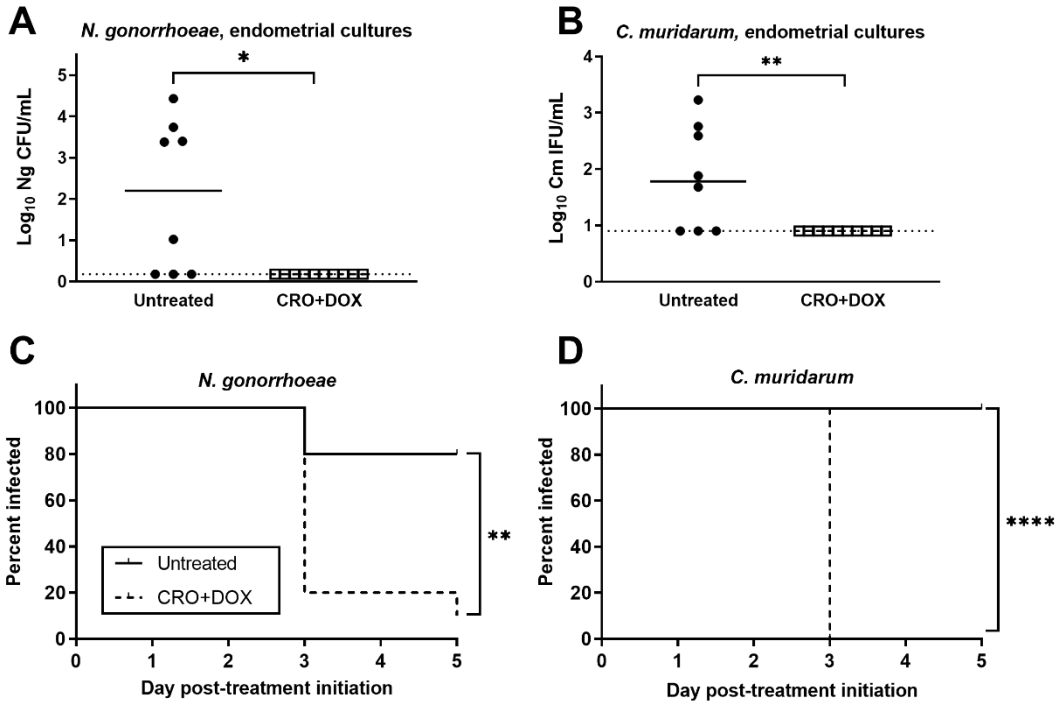

Figure 18. *In vivo* efficacy of currently recommended antibiotics for gonococcal/chlamydial coinfection

Mice were inoculated with *N. gonorrhoeae* and *C. muridarum* simultaneously as described in Fig. 12 and treated with ceftriaxone and doxycycline five days later, or PBS. Results shown are from two independent infections with a total of 8-10 mice per group. (A, B) Endometrial bioburden detected on day 5 post-treatment (day 10 post-bacterial inoculation), expressed as (A) CFU/ml for *N. gonorrhoeae* and (B) IFU/ml for *C. muridarum*. A significant difference in the number of *N. gonorrhoeae* CFU ($p = 0.011$) and *C. muridarum* IFU ($p = 0.007$) was detected in the treated versus untreated groups (Mantel-Cox (log-rank) test). (C, D) Percent of mice with positive vaginal cultures prior to treatment (day 0) and on days 3 and 5 post-treatment for (C) *N. gonorrhoeae* and (D) *C. muridarum*. (*N. gonorrhoeae*, $p = 0.002$ and *C. muridarum*, $p < 0.0001$ compared to untreated controls; Mann-Whitney test). Solid circles and lines represent untreated mice; open squares and dotted lines represent mice treated with ceftriaxone and doxycycline.

to immune responses to chlamydial infection, based on the fact that we do not see the pro-inflammatory cytokines and cellular responses to chlamydial infection that have been previously shown in mice not given estradiol (44, 159, 194). The inflammatory response to *N. gonorrhoeae* and *N. gonorrhoeae*/*C. muridarum* coinfected mice was also subdued in this model. This result differs from many previous reports from our laboratory in which a significant pro-inflammatory cytokine response and vaginal PMN influx were detected in *N. gonorrhoeae*-infected estradiol-treated BALB/c mice, reviewed in reference (92). These previous studies, as well as the previous *N. gonorrhoeae*/*C. muridarum* coinfection model (210) utilized a water-soluble conjugated form of 17-β–estradiol (estradiol$_{ws}$), which did not cause sustained high levels of serum estradiol (92). Our decision to use 17β-estradiol sodium benzoate in the experiments reported here was due to the discontinuation of a nontoxic formulation of the estradiol$_{ws}$ product. Although 17β-estradiol sodium benzoate does allow sustained *N. gonorrhoeae* infection, it also results in higher levels of serum estradiol, which remains elevated for multiple days. Previous data suggest that the estradiol$_{ws}$ formulation was less immunosuppressive than estradiol sodium benzoate formulation used here, based on high MIP-2 levels and a corresponding PMN influx in estradiol$_{ws}$-treated mice infected with *N. gonorrhoeae*, *C. muridarum* or *N. gonorrhoeae* and *C. muridarum* (45, 147, 210). Future refinements of the URT coinfection model will utilize Premarin, which is more similar to the estradiol$_{ws}$ formulation in that it results in physiological estradiol levels within 24 hours post-administration (E. L. Raterman, submitted), and thus may have less of an effect on host inflammatory responses.

One of the primary findings in this model is that the chlamydial bacterial burden was reduced in the presence of gonococcal infection in simultaneously inoculated mice, but not in mice that were infected with *N. gonorrhoeae* before inoculating with *C. muridarum*. We previously showed that establishment of *C. muridarum* infection prior to inoculation with *N. gonorrhoeae* resulted in increased recovery of gonococci compared to mice inoculated with *N. gonorrhoeae* alone, possibly due to lower levels of cationic antimicrobial peptides in *C. muridarum*-infected mice at the time of *N. gonorrhoeae* inoculation (210). There are several differences between the conditions of the coinfection model described previously and the one described here, any combination of which could be responsible for the differences that we see. First, hTf was not used in the previous coinfection model, and in addition to supporting *N. gonorrhoeae* colonization of the URT, hTf supplementation also results in elevated LRT colonization, due to the presence of a host-restricted iron source. Second, in the previously described coinfection model, *C. muridarum* was inoculated prior to estradiol treatment and inoculation with *N. gonorrhoeae,* in order to circumvent the problem of estradiol-treated mice not being susceptible to *C. muridarum*. Interestingly, the effect of enhanced *N. gonorrhoeae* colonization was also increased when the time between *C. muridarum* inoculation and *N. gonorrhoeae* inoculation was lengthened, suggesting that the factor responsible becomes stronger later in *C. muridarum* infection. Given that information, it is not surprising that when the two organisms are inoculated simultaneously, we do not see higher numbers of *N. gonorrhoeae* in coinfected mice. Taken together, the differences in the outcome of coinfection suggests that the order of exposure and the stage of infection in which coinfection is established are important factors that can impact colonization of one or the

other pathogen, perhaps because the host response to infection plays a role. Future studies using mice in which various components of the host response are reduced or absent may help to elucidate these mechanisms.

Finally, the emergence of resistance to the extended-spectrum cephalosporins in *N. gonorrhoeae* has spurred a major research effort towards gonorrhea drug development and the preferred product characteristics for new antibiotics against gonorrhea include activity against *C. trachomatis* (6). Models of URT infection are needed to provide a system for pre-clinical testing of new antibiotics due to possible pharmacokinetic differences at different body sites. Clinical efficacy trials for antibiotics against PID are limited (43, 229), and in one trial, treatment failures with doxycycline and a second-generation cephalosporin were only associated with gonococcal PID (211). Our finding that a 15 mg/kg dose of ceftriaxone was insufficient to clear *N. gonorrhoeae* infection in all mice was unexpected based on previous results showing that that dose was 3 times greater than the minimum dose required for 100% clearance in *N. gonorrhoeae* in the LRT infection model (36). Whether the lower efficacy is due to increased *N. gonorrhoeae* colonization that occurs with hTf supplementation remains to be determined and could have important implications for the development of anti-infectives against gonococcal PID.

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Disclaimer

The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense, the Uniformed Services University of the Health Sciences, or the National Institutes of Health (NIH).

CHAPTER 4: Discussion

SUMMARY OF FINDINGS AND RELEVANCE

Pelvic inflammatory disease is a serious and common complication of *N. gonorrhoeae* and *C. trachomatis* infections that can result in severe sequelae including infertility, ectopic pregnancy, and chronic pain. An estimated 2.5 million women in the US have a history of PID (98), and the associated annual health care costs are over $2 billion (196). Rising numbers of *C. trachomatis* and *N. gonorrhoeae* infections suggest that PID will only become a larger problem in the future, particularly as antibiotic resistance grows and treatment options for *N. gonorrhoeae* become scarcer. Despite how common this condition is, much remains unknown about disease pathology, susceptibility, effectiveness of treatments, and what components of the immune response are helpful or harmful. The lack of animal models for gonococcal PID has prevented answering many of these questions; however, the recent development of the hTf-supplemented mouse model of *N. gonorrhoeae* URT infection provides an avenue for addressing some of these knowledge gaps. In the first studies described in this work, we further characterized the host response in the hTf model and sought to address additional questions about the use of the model, including the effect of different routes of inoculation and the influence of bacterial burden on the immune response. We found that gonococcal infection induces a strong pro-inflammatory cytokine response in the hTf model, regardless of whether bacteria are inoculated transcervically or allowed to ascend to the URT naturally, and that this response, as well as IL-17 and IL-22, was strongly correlated with the vaginal bacterial burden. We examined T cell responses in the context of URT infection, and found that while no significant changes in T cell subsets were

associated with infection, there was a strong negative correlation between Th1 and Th2 cells and the vaginal bacterial burden. Consistent with previously described mechanisms of immunosuppression (112), depletion of TGF-β accelerated clearance of infection. These results strengthen the evidence for a TGF-β-dependent immunosuppressive pathway in that we showed it also occurs in an improved model that provides a host-restricted iron source. Intriguingly, TGF-β depletion during URT infection initiated by transcervical inoculation did not hasten clearance of infection, suggesting that either the host response to transcervically-inoculated bacteria is different, or that the protective response developed during TGF-β depletion is not effective in clearing bacteria from the URT.

In the second study described herein, we expand the potential uses of the hTf model by establishing a model for gonococcal/chlamydial URT coinfection. We describe methods for coinfecting mice with both organisms simultaneously, as well as *N. gonorrhoeae* first followed by *C. muridarum*. We then used this newly-developed methodology to further characterize infection dynamics and host responses during simultaneous coinfection. We found that approximately 88% of mice have *N. gonorrhoeae*/*C. muridarum* coinfection at 10 days post-inoculation. Contrary to expectations based on the previous LRT coinfection model, we observed no significant increase in *N. gonorrhoeae* colonization due to chlamydial coinfection; instead, we found that coinfection seemed to decrease the *C. muridarum* bacterial burden when mice were inoculated with both pathogens simultaneously but not when infected with *N. gonorrhoeae* prior to *C. muridarum* inoculation. These differences may be explained by

order of exposure; the model described by Vonck *et al.* (210) established *C. muridarum* infection prior to inoculating with *N. gonorrhoeae*.

Despite robust infections with both pathogens, we observed only a mild inflammatory response in *N. gonorrhoeae*-only and *N. gonorrhoeae*/*C. muridarum* coinfected mice, with little to no inflammation in mice infected with only *C. muridarum*. Similarly, we found no significant changes in local T cells associated with infections with either or both pathogens. Lastly, we demonstrate a potential use of the coinfection model by testing the efficacy of the current CDC-recommended antibiotic treatment for suspected gonococcal and/or chlamydial PID and find that although the treatment fully cleared all *N. gonorrhoeae* and *C. muridarum* from the URT, it was surprisingly not sufficient to clear all *N. gonorrhoeae* from the LRT.

Through this work we have substantially expanded our knowledge about and potential uses of the hTf mouse model by characterizing host responses in the hTf model and factors governing these responses; additionally, we adapted the model for the purpose of studying gonococcal/chlamydial coinfections, which are prevalent and were previously impossible to study in an animal model.

ASCENDING INFECTION VERSUS TRANSCERVICAL INOCULATION

To our knowledge, the studies described here are the first to compare gonococcal URT infections initiated by transcervical inoculation to those caused by naturally ascending infection. Little is known about the process of bacterial ascension to the URT in humans. Various mechanisms have been proposed; some hypothesize that ascension is facilitated by sperm, to which *N. gonorrhoeae* can bind (86); others suggest that retrograde menstruation may be the culprit (85), an explanation that would make sense

with the timing of acute PID developing during and just after menses. However, it could be that no special circumstances or pathogenic mechanisms are required. Zervomanolakis and colleagues used radiolabeled microspheres to demonstrate that upward transport from the vagina to the uterus occurs during the follicular and luteal phases of the menstrual cycle, and transport to the oviducts occurs during the follicular phase (232). In this study, transport to the uterus was observed in 100% of women and to the fallopian tubes in 79% of women. There may be similar mechanisms for upward transport in the murine reproductive tract, but to our knowledge, no similar studies have been conducted in mice. Given this information, it is somewhat surprising that only 10-20% of women with cervicitis develop URT infection (61), suggesting factors in addition to access to the endometrium play a role in susceptibility to URT infection. If, in fact, transport to the URT is not the limiting factor in producing URT infections, then future studies should focus on identifying host and bacterial factors that are responsible for allowing infections to take hold in the URT. The hTf-supplemented mouse model provides an avenue for such studies.

One limitation of the model in its current form is that we are unable to identify URT infection in live animals, or to monitor URT infection over time in a single animal, as our culture methods require euthanizing the mice and removing the reproductive tracts. Future studies using *in vivo* imaging techniques would allow us to determine the timing of infection ascension, which may allow one to look for correlations between elements of the host response and ascension. The drawback to this approach would of course be that *in vivo* imaging technology offers only semi-quantitative assessment of the URT bacterial burden; however given the wide range of bacterial burdens observed in the URT

(between 10 and 10^4 CFU per mouse), this approach could be sufficient to identify differences.

A classic bacterial genetics approach could also be used to identify bacterial factors that allow ascension or survival in the URT, by using an inoculum consisting of a transposon mutant library, and sequencing the bacterial population in the LRT and URT at different time points following the inoculation with the goal of identifying transposon mutants that were not detected in the URT.

INFLUENCES OF INOCULATION ROUTE AND BACTERIAL BURDEN ON CYTOKINE PROFILE

Another novel aspect of our studies was examining the cytokine profile in relation to the bacterial burden, for mice inoculated vaginally and transcervically. We found a similar profile early in infection for both vaginal and transcervical inoculation, with a drastic but temporary elevation of cytokines associated with inflammation and granulocyte and monocyte recruitment and proliferation (GM-CSF, Gro-α/KC, and IL-6) one day post-inoculation. Other cytokines including RANTES, TNF-α, MIP-1α, MIP-1β, and MIP-2 remained elevated for 3 − 7 days. These findings somewhat recapitulate the findings of Islam and colleagues (85), who inoculated mice transcervically with *N. gonorrhoeae* at different stages of the estrus cycle and followed the inflammatory response for 18 hours post-inoculation. They also observed increases in the expression of GM-CSF, Gro-α /KC, IL-1β, TNF-α, and MIP-1α, though interestingly GM-CSF and TNF-α were only increased in mice that were inoculated during diestrus, while they were elevated in our model despite the estrus-like state induced by estradiol treatment. Additionally, they found that IL-6 was elevated in serum but not local tissue, which

contrasts with our observation. There are several differences in methodology that could explain these discrepancies. Islam and colleagues used FVB mice and 10^7 CFU of *N. gonorrhoeae* strain MS11, while we used BALB/c mice and 10^6 CFU of strain FA1090; they also quantified cytokine expression in tissue homogenates while we used vaginal lavages.

Correlating the bacterial burden with cytokine levels allows us to determine whether each cytokine is induced maximally even by low levels of infection (as is the case for IL-6 and GM-CSF), or whether cytokine expression increases with more severe infections (most other cytokines). Unfortunately, due to the desire to assess the cytokine response at multiple time points as infection progressed, we could not directly determine how the bioburden in the URT affects cytokine production because URT culture is a terminal procedure. However, by determining whether there is a correlation with the LRT bacterial burden, we can make some educated guesses about the role of the URT in inducing that each cytokine. For example, IL-1β is a pro-inflammatory cytokine that is significantly elevated in both transcervically and vaginally inoculated mice; it is also correlated with the vaginal bacterial burden in vaginally inoculated but not transcervically inoculated mice, suggesting that the cells producing this response may be found throughout the reproductive tract (see Fig. 19 for illustration). In contrast, another pro-inflammatory cytokine TNF-α is correlated with the vaginal bacterial burden in both groups of mice, suggesting that it may be elicited by cells only in the LRT.

It is unclear what host mechanisms are responsible for the link between LRT bacterial burden and inflammation. TLR-4, which recognizes gonococcal LOS, seems a

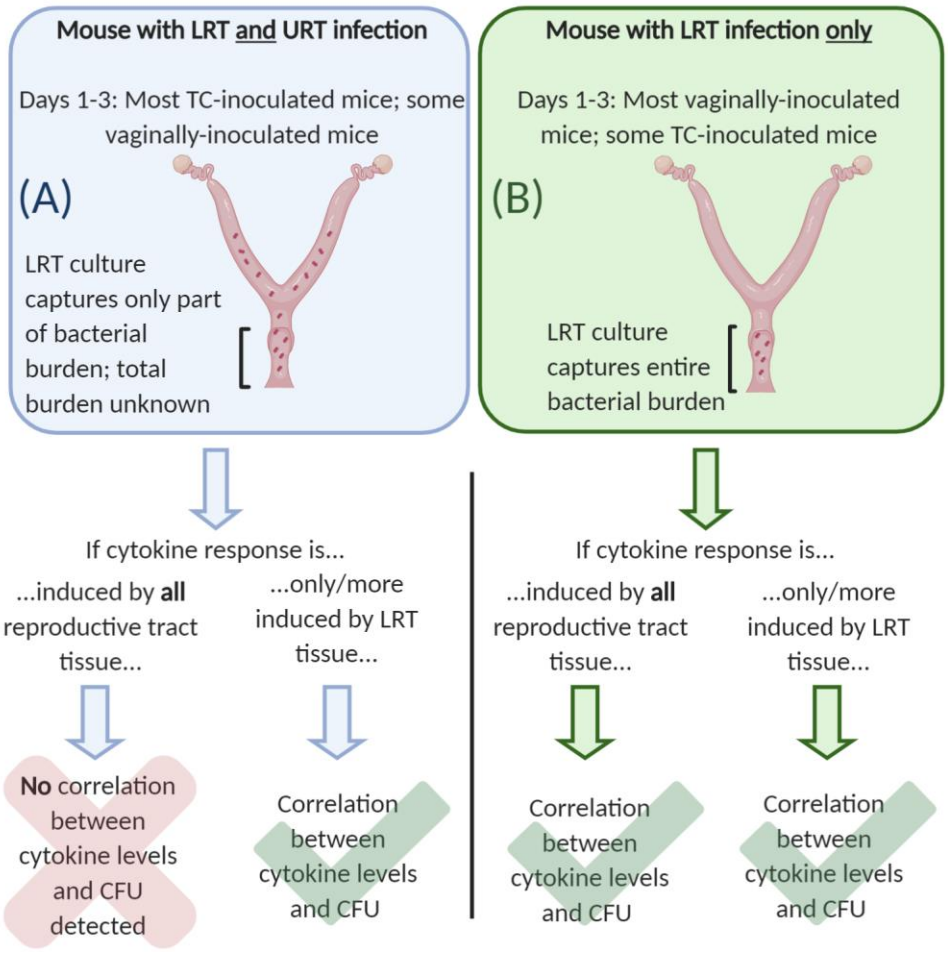

Figure 19. Cytokine correlation with bacterial burden during LRT+URT infection versus LRT infection alone

Shown are two possible scenarios, comparing mice with URT infection in addition to LRT infection (A) with mice that have LRT infection only (B). We have never observed a case of URT infection without LRT infection. About 60-70% of transcervically-inoculated mice have detectable URT infection 1 day after inoculation; by 5 days after inoculation, both vaginally- and transcervically-inoculated mice have similar numbers of URT bacteria, though the timing of ascension is unknown. The scenarios shown at the bottom of the figure represent possible result from correlation analysis of LRT bacteria burden with cytokine levels. Most cytokines from our analysis show correlation with bacterial burden for vaginally-inoculated but not transcervically-inoculated mice – left-hand scenario for both A and B – but some show correlation for both. See Table 1 for information on individual cytokines.

likely candidate for playing a role in this process. Expression and activity of TLR-4 has been demonstrated in vaginal and uterine tissue in mice (185, 199), but to our knowledge the relative expression in the two sites has not been determined. In the future, measurements of cytokine and PRR expression in tissue from each region of the reproductive tract may help to clarify this issue.

Intriguingly, many cytokines were not significantly elevated when comparing group means due to wide variations in cytokine levels between individual mice, but were significantly positively correlated with bacterial burden. Cytokines in this group include IFN-γ, Eotaxin, IL-2, IL-10, IL-17, IL-22, and IP-10. Of this group, only IL-10, IL-17, and IL-22 were previously linked to gonococcal infections (172). To our knowledge, our approach of connecting colonization with cytokine levels at specific time points in individual animals is a novel one, and overall, our findings are consistent with increased PAMP-mediated signaling causing a greater response. Our findings may have a practical use: for *N. gonorrhoeae*, as with *C. trachomatis*, inflammation – particularly high levels of TNF-α – is responsible for oviduct pathology (104), and thus determining factors related to increased inflammation is an important area of research. The finding that certain markers of inflammation are linked to bacterial burden is thus an important step forward and could lead to improved medical care such as initiating additional screening for PID based on a high *N. gonorrhoeae* bacterial burden in the LRT.

T CELL RESPONSE TO LRT AND URT INFECTION

In this work, we did not observe any differences in the average number or percentage of Th1, Th2, Th17, or Tregs induced by *N. gonorrhoeae* infection, including when the data were grouped based on route of inoculation (transcervical versus vaginal),

whether or not the mice were infected, or whether the mice had LRT and URT infection versus only LRT infection. Intriguingly, despite the lack of differences between group averages, we did note a negative correlation between the mean LRT CFU and (1) the number of CD4 cells, and (2) the number and percentage of Th1 and Th2 cells. We also found a negative correlation between the number and percentage of Tregs and URT CFU. Neither LRT or URT bacterial burdens were correlated with the number or percentage of Th17 cells.

Our observation that Th1 and Th2 cells were negatively correlated with bacterial burden is reminiscent of previous studies by Liu and colleagues that *N. gonorrhoeae* infection caused a decrease in Th1 and Th2 cells, though curiously they found that this was linked to an increase in Th17 cells, while we did not observe any increase in Th17 cells (112). The lack of a Th17 response to infection was unexpected since Liu and others (53) had reported that *N. gonorrhoeae* infection was characterized by a Th17 response. However, our findings are consistent with our observation that Th-17 related cytokines IL-17, IL-22, and IL-23 were not significantly elevated (Table 2).

Logically, there are three possible explanations for the observed negative correlation between Th1 and Th2 cells and bacterial burden: (1) that *N. gonorrhoeae* is actively decreasing the number and percent of Th1 and Th2 cells and this effect is increased with higher numbers of bacteria; (2) that there is natural variance in the number of Th1 and Th2 cells in mice, and during infection, animals with naturally higher levels of T cells are more able to control infection, thus decreasing the bacterial burden; or (3) that a third factor is responsible. From the data we currently have, it is not possible to determine the correct explanation with any certainty; we can only speculate. The fact that

we do not see a significant difference in the number of T cells when comparing uninfected mice to infected mice suggests that the "natural variation" hypothesis is more likely than active suppression. On the other hand, evidence for active suppression of CD4 cells in humans with gonorrhea has been reported (11), and *N. gonorrhoeae* binding to CEACAM1 has been shown to suppress activation and proliferation of human T cells (19). However, Opa/CEACAM binding is specific to human cells (103), and thus that mechanism cannot explain our observations. Based on our findings and those of Liu and colleagues, it seems likely that some other as-yet unidentified and non-host-restricted mechanism could be responsible for the suppression of T cell responses in mice. Several approaches could be used to address this matter in future studies. Depleting CD4 T cells during infection would determine the relative contribution of T cells in controlling infection; more specific studies targeting specific T cell subsets would also be useful for similar purposes. Another approach would involve a dose response study, in which mice are inoculated with increasing amounts of *N. gonorrhoeae* to determine whether deliberately increasing or decreasing the bacterial burden of *N. gonorrhoeae* influences T cell numbers.

Finally, the apparent negative correlation between Tregs and URT bacterial burden is somewhat different from the findings of Imarai and colleagues, who found an increase in FOXP3+ Tregs after *N. gonorrhoeae* infection in mice (83). Our methods for infection differ substantially from those described in that work, in which mice were initially infected, and then injected with additional *N. gonorrhoeae* intraperitoneally seven days prior to sacrificing the mice and measuring T cells. Liu and colleagues (110) also found that *N. gonorrhoeae* infection caused an increase in regulatory T cells, but the

Tregs they identified were Tr1 cells, which are a different subset that express IL-10 and not FOXP3. In our experiments, we did not stain cells for IL-10 and thus were unable to quantify this Treg subset. Future studies would ideally include staining for both FOXP3 and IL-10 to determine whether Tr1 cells are induced by URT infection as observed in LRT infection.

TGF-β DEPLETION IN THE CONTEXT OF URT INFECTION

To further explore *N. gonorrhoeae*-induced immunosuppression in the hTf infection model, we chose to test the effect of depleting TGF-β during infection. TGF-β is an immunosuppressive cytokine that was previously shown to be elevated during *N. gonorrhoeae* infection (112). These investigators also showed that antibody-mediated depletion of this cytokine altered the immune response by decreasing the Th17 response and increasing the Th1 and Th2 responses. Importantly, this reversal of the immunosuppression resulted in an antibody response that hastened infection clearance and allowed development of a memory response. Our results were like those reported previously in that we also saw significantly faster infection clearance in vaginally-inoculated mice; however, this was not the case for transcervically-inoculated mice. We also noted that even vaginally-inoculated mice took longer to clear the infection, with 9 days required for 100% clearance compared to the 6 days previously reported. This could be due to our use of hTf supplementation, which increases the LRT bacterial burden in addition to allowing URT infection. Moreover, in one experiment, we attempted to re-infect mice transcervically one month after clearing the initial infection during TGF-β depletion, to determine if there was immune memory; we did not observe any differences in infection rate or clearance for this secondary infection (data not shown). However, the

numbers of mice were small (4 mice per group) and several mice were not infected at all; thus, it is unclear whether these results truly reflect a lack of a memory response.

The difference in the outcome of TGF-β depletion with respect to the route of inoculation raises many questions. Is there a body-site specific difference in the T cell response at the time of TGF- β depletion? Or is the response produced simply insufficient to clear URT infection? Interpretation of our data is further complicated by the fact that under normal infection conditions, a majority (~68%) of hTf-supplemented vaginally-inoculated mice do have ascending infection within 5 days of inoculation. The fact that there is a difference between the groups despite the occurrence of ascending infection in the vaginal inoculation group suggests that the time period between inoculation and ascension is important for the outcome. Additionally, our *in vivo* efficacy data for ceftriaxone in the URT coinfection model imply that infection clearance in the context of hTf supplementation may be different than without supplementation, since the dose used should have been sufficient to clear *N. gonorrhoeae* infection, but was not fully effective. Whether this also holds true for immune-mediated clearance as well as for antibiotic-mediated clearance remains to be seen.

Further studies should determine the T cell profiles of TGF-β-depleted mice in a repeat experiment to determine whether the immune response in transcervically-inoculated mice differs in a way that would explain the lack of clearance upon TGF-β depletion. An additional complementary approach would be to use other methods of artificially encouraging a Th1 response, such as co-administration of IL-12 and/or depletion of IL-10, both of which have also been shown to hasten infection clearance (172).

EFFECT OF *N. GONORRHOEAE*/*C. MURIDARUM* COINFECTION ON *C. MURIDARUM* COLONIZATION

One significant finding from our coinfection experiments was that the *C. muridarum* bacterial burden was decreased in simultaneously coinfected mice, but the *N. gonorrhoeae* bacterial burden was unaffected. This contrasted with previous findings from our laboratory using the LRT coinfection model, in which coinfection increases the gonococcal burden but does not affect chlamydial infection (210). Several possible differences between the model could account for this discrepancy. First, in the model described in this work, both organisms are inoculated simultaneously, while in Vonck's model, *C. muridarum* is inoculated first, followed by *N. gonorrhoeae*. Vonck also observed that lengthening the time of *C. muridarum* infection before introducing *N. gonorrhoeae* resulted in a greater colonization increase; it is plausible that whatever is responsible for the effect develops later in *C. muridarum* infection. Second, as mentioned before, hTf treatment increases gonococcal colonization; it is possible that any advantage to *N. gonorrhoeae* produced by chlamydial infection is overwhelmed by the greater colonization during hTf treatment.

Regarding the decrease in *C. muridarum* burden in coinfected mice, we initially hypothesized that the inflammation produced in response to *N. gonorrhoeae* infection may also target *C. muridarum*, thus decreasing the bacterial burden. This hypothesis was not clearly supported by the data, since only mild inflammation occurred in any group, although there was slightly more pronounced inflammation in the coinfected group than the *C. muridarum*-only group. Additionally, *C. muridarum* thrives during the diestrus stage of the estrous cycle when neutrophils are abundant (48, 163); it would thus be surprising if an increase in neutrophils was detrimental to *C. muridarum* infection, but

another factor could be responsible. Alternative hypotheses to explain this effect include the possibility of competition for resources between *N. gonorrhoeae* and *C. muridarum*, or *N. gonorrhoeae* somehow blocking *C. muridarum* EB from entering cells.

In the future, expanding and further characterizing the URT coinfection model to include all orders of inoculation may help to address these questions. If the order of inoculation is the important factor in determining whether coinfection is beneficial to *N. gonorrhoeae*, we would expect to recover higher numbers of *N. gonorrhoeae* from hTf-supplemented mice that were pre-infected with *C. muridarum*, at least in the LRT as in the LRT coinfection model. A *C. muridarum*-first model would also help to clarify the issue of the *C. muridarum* bacterial burden, as we could determine whether the decrease in *C. muridarum* colonization took place at the same time as inflammation following *N. gonorrhoeae* inoculation or whether it was dependent on some factor that took longer to develop. Future studies should also be undertaken to define host responses and the milieu of the genital tract after primary inoculation with *N. gonorrhoeae* or *C. muridarum* so that hypotheses can be formulated to explain how one pathogen might affect the colonization ability of the other. The use of URT tissue may be helpful here, as my attempts to quantify expression of antimicrobial factors in the LRT in using the LRT coinfection model were unsuccessful, due in part to low amounts of mRNA in the squamous cells that form the superficial tissue of the LRT during estradiol treatment or natural estrus.

WEAK INFLAMMATORY RESPONSE IN COINFECTION MODEL

Conspicuous only by its absence, the inflammatory response in the coinfection model was one of our disappointing findings. We observed little to no PMN influx, low

levels of pro-inflammatory cytokines in all groups but especially the *C. muridarum*-only group, and no significant T cell or antibody response. The subdued nature of the immune response in this model is likely due to the formulation of the estradiol we used, which was 17-β-estradiol sodium benzoate suspended in sesame oil, given subcutaneously. Other recent work from our laboratory showed that this formulation of estradiol results in serum estradiol levels that are several thousand-fold greater than those induced by water soluble formulations (E. L. Raterman, submitted), including the now-unavailable estradiol that was used in many of the previous mouse studies from our laboratory, and Premarin, which is the formulation used in the studies described in Chapter 2. Raterman also showed that mice treated with oil-soluble estradiol have significantly reduced inflammatory responses during *N. gonorrhoeae* infection. This finding is consistent with other previous reports of estradiol suppressing inflammation in the gut (186) and with the reports of Islam (85) that mice were less responsive to *N. gonorrhoeae* inoculation when estradiol-treated or in estradiol-dominant stages of the estrus cycle.

The requirement for estradiol treatment is a significant drawback to this animal model, though currently an unavoidable one. Recent pilot studies from our laboratory have tested whether hTf supplementation may support infection of mice not given estradiol. Preliminary results suggest that 50% of hTf-supplemented mice can be infected for at least 7 days without exogenous estradiol, but by the transcervical route only (M. Pilligua-Lucas and AE Jerse, unpublished observations). Although a 50% infection rate is less than ideal, it is feasible that this could be used to investigate the immune response to *N. gonorrhoeae*, as well as *N. gonorrhoeae*/*C. muridarum* coinfections without the immunosuppressive effect of estradiol. Another alternative is conducting the coinfection

studies using Premarin, which although still immunosuppressive, is significantly less so than the oil-soluble estradiol and may more accurately reflect natural infections. The drawback to this approach is that *N. gonorrhoeae* infection is not sustained for as long with Premarin, hence our decision to use oil-soluble estradiol prior to realizing that the effect on the immune response was much more drastic.

ANTIBIOTIC TREATMENT IN URT COINFECTION MODEL

Because the immune response to coinfection is so weak in the model we describe in this work, we realize that it is not an ideal model for investigating disease parameters such as tubal damage, which is primarily due to immunopathology for both pathogens (46, 104). However, we found it is a robust and reliable model for establishing long-lasting infection with both *N. gonorrhoeae* and *C. muridarum*, and is therefore well-suited for applications such as testing microbicides and antibiotics that have dual activity against both pathogens. To demonstrate this potential application of the coinfection model, we tested the efficacy of ceftriaxone and doxycycline, which are a CDC-recommended PID treatment regimen (29). We began treatment 5 days after infection was established to have pre-treatment culture time points, using dosages previously demonstrated to be effective in mice, and sacrificed mice after 5 days of treatment to perform URT cultures. We found that the doxycycline treatment was sufficient to clear *C. muridarum* infection in 100% of mice, in both the LRT and the URT. However, treatment was not sufficient to clear *N. gonorrhoeae* from the LRT, in contrast to previous findings from our lab that this dose of ceftriaxone was 3-fold greater than the minimum dose needed to clear infection in all mice (36). This finding suggests that either hTf supplementation or the presence of URT infection reduces the effectiveness of antibiotic

treatment and yields an interesting and important avenue for future studies. Haggerty and colleagues reported persistent endometritis 30 days after completion of cefoxitin and doxycycline treatment in 43% of women with PID, while 8% and 10% remained positive for *N. gonorrhoeae* and *C. trachomatis*, respectively; rates of treatment failure were even higher in other groups (66). Unfortunately, other studies on PID treatment efficacy are scarce; it may be that higher doses or longer treatments are required to clear infections that are present in the URT as well as LRT. Thus, our study supports an important future application for the hTf supplementation model, as well as the coinfection model.

CONCLUSIONS

In the work described here, I have expanded upon potential uses of hTf-supplemented models of URT infection. In my first research chapter, I describe the immune response to *N. gonorrhoeae* in the context of this model, with an emphasis on the local inflammatory response and the CD4 T cell response to infection. By comparing infections initiated by transcervical inoculation versus vaginal inoculation, my work demonstrated that both methods are viable options for future uses of the model; further, the use of both methods yielded several intriguing findings that hint toward differing contributions of the upper and lower reproductive tracts to the host response to infection. Notably, the local inflammatory response to infection was slightly stronger and more sustained in vaginally-inoculated mice. Further, I showed for the first time that inflammation in response to *N. gonorrhoeae* infection is apparently determined by the bacterial burden, and some markers of inflammation are only elevated in mice with particularly high bacterial burdens. This phenomenon is primarily seen in vaginally- and

not transcervically-inoculated mice, perhaps because the vaginal swabs used to measure infection do not fully capture the *N. gonorrhoeae* bioburden in transcervically-inoculated animals. This finding is significant both for future animal studies, which should utilize similar analyses in order to ensure that they do not miss elements of the host response that may only occur in animals with high levels of colonization, as well as human studies which may benefit from examining colonization load as a factor in whether individuals are symptomatic or at higher risk for complications.

The CD4 T cell response to *N. gonorrhoeae* infection in this model was also strongly correlated with the bacterial burden of individual animals, with markedly different trends associated with higher colonization in the LRT versus the URT. Significantly, a decrease in both the number and percentage of Th1 and Th2 cells was linked to increased colonization of the LRT, reminiscent of previous findings in the LRT infection model (109, 112). Depletion of TGF-β, which had been previously shown to prevent suppression of the Th1 and Th2 responses and precipitate rapid infection clearance, was only effective in vaginally-inoculated mice. These results raised the question of whether the TGF-β-depletion-induced protective immune response to URT infection is not effective after transcervical inoculation, or whether it is not induced in transcervically-inoculated animals in the first place. The new avenues of research opened by these data should contribute greatly to our understanding of *N. gonorrhoeae* URT infections in particular, and infections and immunology of the reproductive tract in general.

In my second research chapter, I describe an expansion of the hTf-supplemented model in which mice can be coinfected with *N. gonorrhoeae* and *C. muridarum*. The

major break-through which allowed this work was my discovery that transcervical inoculation of *C. muridarum* allows us to bypass the resistance to infection caused by estradiol treatment in mice. Using this technique, I developed methods for establishing coinfection with both organisms simultaneously, and with *N. gonorrhoeae* followed by *C. muridarum*. Neither orders of infection were possible with the previously-established coinfection model, in which mice had to be inoculated with *C. muridarum* first, followed by *N. gonorrhoeae*. In subsequent studies using the simultaneous inoculation procedure, I showed that robust infection with both organisms is sustained for at least ten days in the LRT and URT; longer infections may be possible by extending the duration of hTf supplementation. Unexpectedly, *C. muridarum* colonization was negatively impacted by the presence of *N. gonorrhoeae* coinfection, although this was only the case for simultaneously-inoculated mice. Further studies may determine why coinfection has this effect on *C. muridarum*, perhaps by utilizing knock-out mice to isolate components of the host response that may influence *C. muridarum* growth, such as IFN-γ or PMNs. Surprisingly, despite reasonably high levels of colonization with both organisms, I found that the immune response to infection was subdued, rarely significantly elevated over uninfected controls. Concurrent studies by others in our lab demonstrated that the lack of an immune response was likely due to the formulation of estradiol used in these studies, which results in far higher levels of serum estradiol than found in naturally cycling mice. In the future, this coinfection model may be improved by using different formulations of estradiol, or no estradiol, to avoid the immunosuppression evidenced here; such an improved model would be far more useful for vaccine studies or studies on the host response to coinfection. Furthermore, a model with a more substantial host response

would be useful to test the hypothesis that the decreased chlamydial bioburden in coinfection is due to some component of the host response; if that is the case, we would expect to see a stronger effect, with a greater difference in the chlamydial colonization load between coinfected mice and those with *C. muridarum* alone. Meanwhile, the model I described here is most useful for applications which require higher proportions of infected mice and higher levels of colonization, such as testing antibiotics, as I demonstrated in the final part of my second research chapter.

Overall, the work described here has advanced the field of animal modeling of URT infections, by improving our understanding of factors governing the host response to URT infection, showing that route of inoculation and bacterial burden play important roles in the type of response elicited, and by developing new methods and tools for studying gonococcal/chlamydial coinfections.

REFERENCES

1. Aflatoonian R, Fazeli A. 2008. Toll-like receptors in female reproductive tract and their menstrual cycle dependent expression. *J. Reprod. Immunol.* 77(1):7–13

2. Agostinis C, Mangogna A, Bossi F, Ricci G, Kishore U, Bulla R. 2019. Uterine immunity and microbiota: A shifting paradigm. *Front. Immunol.* 10(OCT):2387

3. Agrawal T, Bhengraj AR, Vats V, Salhan S, Mittal A. 2011. Expression of TLR 2, TLR 4 and iNOS in cervical monocytes of *Chlamydia trachomatis*-infected women and their role in host immune response. *Am. J. Reprod. Immunol.* 66(6):534–43

4. Aiyar A, Quayle AJ, Buckner LR, Sherchand SP, Chang TL, et al. 2014. Influence of the tryptophan-indole-IFNγ axis on human genital *Chlamydia trachomatis* infection: Role of vaginal co-infections. *Front. Cell. Infect. Microbiol.* 4(JUN):72

5. Ajayi AF, Akhigbe RE. 2020. Staging of the estrous cycle and induction of estrus in experimental rodents: an update. *Fertil. Res. Pract.* 6(1):5

6. Alirol E, Wi TE, Bala M, Bazzo ML, Chen XS, et al. 2017. Multidrug-resistant gonorrhea: A research and development roadmap to discover new medicines. *PLoS Med.* 14(7):1–12

7. Altmäe S. 2018. Commentary: Uterine microbiota: residents, tourists, or invaders? *Front. Immunol.* 9:1874

8. Anderson JE, Hobbs MM, Biswas GD, Sparling PF. 2003. Opposing selective forces for expression of the gonococcal lactoferrin receptor. *Mol. Microbiol.* 48(5):1325–37

9. Anderson JE, Leone PA, Miller WC, Chen C ju, Hobbs MM, Sparling PF. 2001. Selection for expression of the gonococcal hemoglobin receptor during menses. *J. Infect. Dis.* 184(12):1621–23

10. Anyalechi GE, Hong J, Kreisel K, Torrone E, Boulet S, et al. 2019. Self-reported infertility and associated pelvic inflammatory disease among women of reproductive age - National health and nutrition examination survey, United States, 2013-2016. *Sex. Transm. Dis.* 46(7):446–51

11. Anzala AO, Simonsen JN, Kimani J, Ball TB, Nagelkerke NJD, et al. 2000. Acute sexually transmitted infections increase human immunodeficiency virus type 1 plasma viremia, increase plasma type 2 cytokines, and decrease CD4 cell counts. *J. Infect. Dis.* 182(2):459–66

12. Aral S, Brunham R, Cates W, Eschenbach DA, Farmer M, et al. 1991. Pelvic inflammatory disease: guidelines for prevention and management. MMWR Recomm Rep

13. Arko RJ. 1989. Animal models for pathogenic *Neisseria* species. *Clin. Microbiol. Rev.* 2(Suppl):S56–59

14. Barron AL, Pasley JN, Rank RG, White HJ, Mrak RE. 1988. Chlamydial salpingitis in female guinea pigs receiving oral contraceptives. *Sex. Transm. Dis.* 15(3):169–73

15. Beagley KW, Gockel CM. 2003. Regulation of innate and adaptive immunity by the female sex hormones oestradiol and progesterone. *FEMS Immunol. Med. Microbiol.* 38(1):13–22

16. Bell JD, Bergin IL, Schmidt K, Zochowski MK, Aronoff DM, Patton DL. 2011. Nonhuman primate models used to study pelvic inflammatory disease caused by *Chlamydia trachomatis*. *Infect. Dis. Obstet. Gynecol.* 2011:1–7

17. Bellofiore N, Cousins F, Temple-Smith P, Dickinson H, Evans J. 2018. A missing piece: The spiny mouse and the puzzle of menstruating species. *J. Mol. Endocrinol.* 61(1):R25–41

18. Biegel Carson SDB, Klebba PE, Newton SMC, Sparling PF. 1999. Ferric enterobactin binding and utilization by *Neisseria gonorrhoeae*. *J. Bacteriol.* 181(9):2895–2901

19. Boulton IC, Gray-Owen SD. 2002. Neisserial binding to CEACAMI arrests the activation and proliferation of CD4+ T lymphocytes. *Nat. Immunol.* 3(3):229–36

20. Brown JL. 2018. Comparative ovarian function and reproductive monitoring of endangered mammals. *Theriogenology*. 109:2–13

21. Brown WJ, Lucas CT, Kuhn US. 1972. Gonorrhoea in the chimpanzee. Infection with laboratory-passed gonococci and by natural transmission. *Br. J. Vener. Dis.* 48(3):177–78

22. Brunham RC, Gottlieb SL, Paavonen J. 2015. Pelvic inflammatory disease. *N. Engl. J. Med.* 372(21):2039–48

23. Buder S, Schöfer H, Meyer T, Bremer V, Kohl PK, et al. 2019. Bacterial sexually transmitted infections. *JDDG J. der Dtsch. Dermatologischen Gesellschaft*. 17(3):287–315

24. Bulletti C, De Ziegler D, Setti PL, Cicinelli E, Polli V, Flamigni C. 2004. The patterns of uterine contractility in normal menstruating women: From physiology to pathology. *Ann N Y Acad Sci*. 2004 Dec;1034:64-83

25. Burnett AM, Anderson CP, Zwank MD. 2012. Laboratory-confirmed gonorrhea and/or chlamydia rates in clinically diagnosed pelvic inflammatory disease and cervicitis. *Am. J. Emerg. Med.* 30(7):1114–17

26. Butler MM, Waidyarachchi SL, Connolly KL, Jerse AE, Chai W, et al. 2018. Aminomethyl spectinomycins as therapeutics for drug-resistant gonorrhea and chlamydia coinfections. *Antimicrob. Agents Chemother.* 62(5): e00325-18

27. Campbell J, Huang Y, Liu Y, Schenken R, Arulanandam B, Zhong G. 2014. Bioluminescence imaging of *Chlamydia muridarum* ascending infection in mice. *PLoS One*. 9(7): e101634

28. Carmichael JR, Tifrea D, Pal S, De la Maza LM. 2013. Differences in infectivity and induction of infertility: A comparative study of *Chlamydia trachomatis* strains in the murine model. *Microbes Infect.* 15(3):219–29

29. Centers for Disease Control and Prevention Sexually Transmitted Diseases Treatment Guidelines: Pelvic Inflammatory Disease. 2015

30. Château A, Seifert HS. 2016. *Neisseria gonorrhoeae* survives within and modulates apoptosis and inflammatory cytokine production of human macrophages. *Cell. Microbiol.* 18(4):546–60

31. Chatwani A, Harmanli OH, Nyirjesy P, Reece EA. 1996. Significance of genital Mycoplasmas in pelvic inflammatory disease: Innocent bystander! *Infect. Dis. Obstet. Gynecol.* 4:263–68

32. Chen H, Wen Y, Li Z. 2019. Clear victory for *Chlamydia*: The subversion of host innate immunity. *Front. Microbiol.* 10:1412

33. Chen JZ, Gratrix J, Brandley J, Smyczek P, Parker P, et al. 2017. Retrospective review of gonococcal and chlamydial cases of epididymitis at 2 Canadian sexually transmitted infection clinics, 2004-2014. *Sex. Transm. Dis.* 44(6):359–61

34. Cohen CR, Manhart LE, Bukusi EA, Astete S, Brunham RC, et al. 2002. Association between *Mycoplasma genitalium* and acute endometritis. *Lancet*. 359(9308):765–66

35. Cohen MS, Cannon JG, Jerse AE, Charniga LM, Isbey SF, Whicker LG. 1994. Human experimentation with *Neisseria gonorrhoeae*: Rationale, methods, and implications for the biology of infection and vaccine development. *J. Infect. Dis.* 169(3):532–37

36. Connolly KL, Eakin AE, Gomez C, Osborn BL, Unemo M, Jerse AE. 2019. Pharmacokinetic data are predictive of in vivo efficacy for cefixime and ceftriaxone against susceptible and resistant *Neisseria gonorrhoeae* strains in the gonorrhea mouse model. *Antimicrob. Agents Chemother.* 63(3):

37. Cornelissen CN, Hollander A. 2011. TonB-dependent transporters expressed by *Neisseria gonorrhoeae*. *Front. Microbiol.* 2(MAY):117

38. Cornelissen CN, Kelley M, Hobbs MM, Anderson JE, Cannon JG, et al. 1998. The transferrin receptor expressed by gonococcal strain FA1090 is required for the experimental infection of human male volunteers. *Mol. Microbiol.* 27(3):611–16

39. Corr TE, Sullivan J, Frazer LC, Andrews CW, O'Connell CM, Darville T. 2014. Steroids alone or as adjunctive therapy with doxycycline fail to improve oviduct damage in mice infected with *Chlamydia muridarum*. *Clin. Vaccine Immunol.* 21(6):824–30

40. Creighton S. 2011. Gonorrhoea. *BMJ Clin. Evid.* 2011:1604

41. Curran JW. 1979. Management of gonococcal pelvic inflammatory disease. *Sex. Transm. Dis.* 6(2):174–80

42. Curry A, Williams T, Penny ML. 2019. Pelvic inflammatory disease: Diagnosis, management, and prevention. *Am. Fam. Physician*. 100(6):357–64

43. Darville T. 2018. Pelvic Inflammatory Disease: identifying research gaps - proceedings of a workshop sponsored by DHHS/NIH/NIAID November 3-4, 2011. *Physiol. Behav.* 176(1):139–48

44. Darville T, Andrews J, Rank RG. 2000. Does inhibition of tumor necrosis factor alpha affect chlamydial genital tract infection in mice and guinea pigs? *Infect. Immun.* 68(9):5299–5305

45. Darville T, Andrews J, Sikes JD, Fraley PL, Braswell L, Rank RG. 2001. Mouse strain-dependent chemokine regulation of the genital tract T helper cell type 1 immune response. *Infect. Immun.* 69(12):7419–24

46. Darville T, Hiltke TJ. 2010. Pathogenesis of genital tract disease due to *Chlamydia trachomatis*. *J. Infect. Dis.* 201 Suppl(S2):S114-25

47. Darville T, O'Neill JM, Andrews CW, Nagarajan UM, Stahl L, Ojcius DM. 2003. Toll-like receptor-2, but not toll-like receptor-4, is essential for development of oviduct pathology in chlamydial genital tract infection. *J. Immunol.* 171(11):6187–97

48. De Clercq E, Kalmar I, Vanrompay D. 2013. Animal models for studying female genital tract infection with *Chlamydia trachomatis*. *Infect. Immun.* 81(9):3060–67

49. Dicker LW, Mosure DJ, Berman SM, Levine WC. 2003. Gonorrhea prevalence and coinfection with chlamydia in women in the United States, 2000. *Sex. Transm. Dis.* 30(5):472–76

50. Edwards JL. 2008. The role of complement in gonococcal infection of cervical

epithelia. *Vaccine*. 26(SUPPL. 8):I56-61

51. Edwards JL, Butler EK. 2011. The pathobiology of *Neisseria gonorrhoeae* lower female genital tract infection. *Front. Microbiol.* 2(MAY):1–12

52. Elwell C, Mirrashidi K, Engel J. 2016. Chlamydia cell biology and pathogenesis. *Nat. Rev. Microbiol.* 14(6):385–400

53. Feinen B, Jerse AE, Gaffen SL, Russell MW. 2010. Critical role of Th17 responses in a murine model of *Neisseria gonorrhoeae* genital infection. *Mucosal Immunol.* 3(3):312–21

54. Feinen B, Russell MW. 2012. Contrasting roles of IL-22 and IL-17 in murine genital tract infection by *Neisseria gonorrhoeae*. *Front. Immunol.* 3(FEB):11

55. Francis IP, Islam EA, Gower AC, Shaik-Dasthagirisaheb YB, Gray-Owen SD, Wetzler LM. 2018. Murine host response to *Neisseria gonorrhoeae* upper genital tract infection reveals a common transcriptional signature, plus distinct inflammatory responses that vary between reproductive cycle phases. *BMC Genomics*. 19(1):627

56. Gagliardi MC, Starnino S, Teloni R, Mariotti S, Dal Conte I, et al. 2011. Circulating levels of interleukin-17A and interleukin-23 are increased in patients with gonococcal infection. *FEMS Immunol. Med. Microbiol.* 61(1):129–32

57. Gangaiah D, Raterman EL, Wu H, Fortney KR, Gao H, et al. 2017. Both MisR (CpxR) and MisS (CpxA) are required for *Neisseria gonorrhoeae* infection in a murine model of lower genital tract infection. *Infect. Immun.* 85(9):e00307-17

58. Geisler WM. 2010. Duration of untreated, uncomplicated *Chlamydia trachomatis* genital infection and factors associated with chlamydia resolution: a review of human studies. *J. Infect. Dis.* 201(S2):104–13

59. Goller JL, De Livera AM, Fairley CK, Guy RJ, Bradshaw CS, et al. 2016. Population attributable fraction of pelvic inflammatory disease associated with chlamydia and gonorrhoea: A cross-sectional analysis of Australian sexual health clinic data. *Sex. Transm. Infect.* 92(7):525–31

60. Gondek DC, Olive AJ, Stary G, Starnbach MN. 2012. CD4 + T Cells are necessary and sufficient to confer protection against *Chlamydia trachomatis* infection in the murine upper genital tract. *J. Immunol.* 189(5):2441–49

61. Gradison M. 2012. Pelvic inflammatory disease. *Am. Fam. Physician*. 85(8):791–96

62. Gray-Owen SD, Schryvers AB. 1993. The interaction of primate transferrins with receptors on bacteria pathogenic to humans. *Microb. Pathog.* 14(5):389–98

63. Gulati S, Pennington MW, Czerwinski A, Carter D, Zheng B, et al. 2019. Preclinical efficacy of a lipooligosaccharide peptide mimic candidate gonococcal vaccine. *MBio*. 10(6): e02552-19

64. Hafner L, Beagley K, Timms P. 2008. *Chlamydia trachomatis* infection: host immune responses and potential vaccines. *Mucosal Immunol.* 1(2):116-30

65. Haggerty CL, Peipert JF, Weitzen S, Hendrix SL, Holley RL, et al. 2005. Predictors of chronic pelvic pain in an urban population of women with symptoms and signs of pelvic inflammatory disease. *Sex. Transm. Dis.* 32(5):293–99

66. Haggerty CL, Totten PA, Astete SG, Lee S, Hoferka SL, et al. 2008. Failure of cefoxitin and doxycycline to eradicate endometrial *Mycoplasma genitalium* and the consequence for clinical cure of pelvic inflammatory disease. *Sex. Transm. Infect.* 84(5):338–42

67. Haggerty CL, Totten PA, Astete SG, Ness RB. 2006. *Mycoplasma genitalium* among women with nongonococcal, nonchlamydial pelvic inflammatory disease. *Infect. Dis. Obstet. Gynecol.* 2006:30184

68. Hart KM, Murphy AJ, Barrett KT, Wira CR, Guyre PM, Pioli PA. 2009. Functional expression of pattern recognition receptors in tissues of the human female reproductive tract. *J. Reprod. Immunol.* 80(1–2):33–40

69. Hawkins SM, Matzuk MM. 2008. The menstrual cycle. *Ann. N. Y. Acad. Sci.* 1135(1):10–18

70. Hedges SR, Sibley DA, Mayo MS, Hook III EW, Russell MW. 1998. Cytokine and antibody responses in women infected with *Neisseria gonorrhoeae*: Effects of concomitant infections. *J. Infect. Dis.* 178(3):742–51

71. Heinonen PK, Miettinen A. 1994. Laparoscopic study on the microbiology and severity of acute pelvic inflammatory disease. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 57(2):85–89

72. Heydarian M, Yang T, Schweinlin M, Steinke M, Walles H, et al. 2019. Biomimetic human tissue model for long-term study of *Neisseria gonorrhoeae* infection. *Front. Microbiol.* 10(JULY):1740

73. Hickey DK, Patel M V., Fahey J V., Wira CR. 2011. Innate and adaptive immunity at mucosal surfaces of the female reproductive tract: Stratification and integration of immune protection against the transmission of sexually transmitted infections. *J. Reprod. Immunol.* 88(2):185–94

74. Hill SA, Masters TL, Wachter J. 2016. Gonorrhea – An evolving disease of the new millennium. *Microb. Cell*. 3(9):371–89

75. Hillier SL, Kiviat NB, Hawes SE, Hasselquist MB, Hanssen PW, et al. 1996. Role

of bacterial vaginosis-associated microorganisms in endometritis. *Am. J. Obstet. Gynecol.* 175(2):435–41

76. Hillis SD, Joesoef R, Marchbanks PA, Wasserheit JN, Cates W, Westrom L. 1993. Delayed care of pelvic inflammatory disease as a risk factor for impaired fertility. *Am. J. Obstet. Gynecol.* 168(5):1503–9

77. Hobbs MM, Anderson JE, Balthazar JT, Kandler JL, Carlson RW, et al. 2013. Lipid A's structure mediates *Neisseria gonorrhoeae* fitness during experimental infection of mice and men. *MBio*. 4(6): e00892-13

78. Hobbs MM, Sparling PF, Cohen MS, Shafer WM, Deal CD, Jerse AE. 2011. Experimental gonococcal infection in male volunteers: Cumulative experience with *Neisseria gonorrhoeae strains* FA1090 and MS11. *Front. Microbiol.* 2(MAY):123

79. Hocking JS, Kong FYS, Timms P, Huston WM, Tabrizi SN. 2014. Treatment of rectal chlamydia infection may be more complicated than we originally thought. *J. Antimicrob. Chemother.* 70(4):961–64

80. Holbein BE. 1981. Enhancement of *Neisseria meningitidis* infection in mice by addition of iron bound to transferrin. *Infect. Immun.* 34(1):120–25

81. Hook E, Handsfield H. 1999. Gonococcal infections in the adult. In *Sexually transmitted infections*, ed K Holmes, PF Sparling, P-A Mardh, W Stamm, P Piot, J Wasserheit, pp. 451–66. New York, NY: McGraw-Hill. 3rd ed.

82. Huang C-C, Huang C-C, Lin S-Y, Chang CY-Y, Lin W-C, et al. 2019. Association of pelvic inflammatory disease (PID) with ectopic pregnancy and preterm labor in Taiwan: A nationwide population-based retrospective cohort study. *PLoS One*. 14(8):e0219351

83. Imarai M, Candia E, Rodriguez-Tirado C, Tognarelli J, Pardo M, et al. 2008. Regulatory T cells are locally induced during intravaginal infection of mice with *Neisseria gonorrhoeae*. *Infect. Immun.* 76(12):5456–65

84. Islam EA, Anipindi VC, Francis I, Shaik-Dasthagirisaheb Y, Xu S, et al. 2018. Specific binding to differentially expressed human carcinoembryonic antigenrelated cell adhesion molecules determines the outcome of *Neisseria gonorrhoeae* infections along the female reproductive tract. *Infect. Immun.* 86(8):1–19

85. Islam EA, Shaik-Dasthagirisaheb Y, Kaushic C, Wetzler LM, Gray-Owen SD. 2016. The reproductive cycle is a pathogenic determinant during gonococcal pelvic inflammatory disease in mice. *Mucosal Immunol.* 9(4):1051–64

86. James-Holmquest AN, Swanson J, Buchanan TM, Wende RD, Williams RP. 1974. Differential attachment by piliated and nonpiliated *Neisseria gonorrhoeae* to

human sperm. *Infect. Immun.* 9(5):897–902

87. Jean S, Juneau RA, Criss AK, Cornelissen CN. 2016. *Neisseria gonorrhoeae* evades calprotectin-mediated nutritional immunity and survives neutrophil extracellular traps by production of TdfH. *Infect. Immun.* 84(10):2982–94

88. Jerse AE. 1999. Experimental gonococcal genital tract infection and opacity protein expression in estradiol-treated mice. *Infect. Immun.* 67(11):5699–5708

89. Jerse AE, Bash MC, Russell MW. 2014. Vaccines against gonorrhea: Current status and future challenges. *Vaccine*. 32(14):1579–87

90. Jerse AE, Cohen MS, Drown PM, Whicker LG, Isbey SF, et al. 1994. Multiple gonococcal opacity proteins are expressed during experimental urethral infection in the male. *J. Exp. Med.* 179(3):911–20

91. Jerse AE, Crow ET, Bordner AN, Rahman I, Cornelissen CN, et al. 2002. Growth of *Neisseria gonorrhoeae* in the female mouse genital tract does not require the gonococcal transferrin or hemoglobin receptors and may be enhanced by commensal lactobacilli. *Infect. Immun.* 70(5):2549–58

92. Jerse AE, Wu H, Packiam M, Vonck RA, Begum AA, Garvin LE. 2011. Estradiol-treated female mice as surrogate hosts for *Neisseria gonorrhoeae* genital tract infections. *Front. Microbiol.* 2(JULY):107

93. Kairys N, Garg M. 2020. *Bacterial Vaginosis*. StatPearls Publishing

94. Keith LG, Berger GS, Edelman DA, Newton W, Fullan N, et al. 1984. On the causation of pelvic inflammatory disease. *Am. J. Obstet. Gynecol.* 149(2):215–24

95. Knapp JS, Clark VL. 1984. Anaerobic growth of *Neisseria gonorrhoeae* coupled to nitrite reduction. *Infect. Immun.* 46(1):176–81

96. Korn AP, Hessol NA, Padian NS, Bolan GA, Donegan E, et al. 1998. Risk factors for plasma cell endometritis among women with cervical *Neisseria gonorrhoeae*, cervical *Chlamydia trachomatis*, or bacterial vaginosis. *Am. J. Obstet. Gynecol.* 178(5):987–90

97. Kraus SJ, Brown WJ, Arko RJ. 1975. Acquired and natural immunity to gonococcal infection in chimpanzees. *J. Clin. Invest.* 55(6):1349–56

98. Kreisel K, Torrone E, Bernstein K, Hong J, Gorwitz R. 2017. Prevalence of pelvic inflammatory disease in sexually experienced women of reproductive age — United States, 2013-2014. *MMWR Morb Mortal Wkly Rep.* 66(3):80-83

99. Kreisel K, Weston E, Braxton J, Llata E, Torrone E. 2017. Keeping an eye on chlamydia and gonorrhea conjunctivitis in infants in the United States, 2010-2015. *Sex. Transm. Dis.* 44(6):356–58

100. Laniewski P, Gomez A, Hire G, So M, Herbst-Kralovetz MM. 2017. Human three-dimensional endometrial epithelial cell model to study host interactions with vaginal bacteria and *Neisseria gonorrhoeae*. *Infect. Immun.* 85(3): e01049-16

101. Laniewski P, Herbst-Kralovetz MM. 2019. Analysis of host responses to *Neisseria gonorrhoeae* using a human three-dimensional endometrial epithelial cell model. *Methods Mol. Biol.* 1997:347–61

102. Lee BC, Schryvers AB. 1988. Specificity of the lactoferrin and transferrin receptors in *Neisseria gonorrhoeae*. *Mol. Microbiol.* 2(6):827–29

103. Lee HSW, Boulton IC, Reddin K, Wong H, Halliwell D, et al. 2007. Neisserial outer membrane vesicles bind the coinhibitory receptor carcinoembryonic antigen-related cellular adhesion molecule 1 and suppress CD4+ T lymphocyte function. *Infect. Immun.* 75(9):4449–55

104. Lenz JD, Dillard JP. 2018. Pathogenesis of *Neisseria gonorrhoeae* and the host defense in ascending infections of human fallopian tube. *Front. Immunol.* 9(NOV):2710

105. Leuzzi R, Serino L, Scarselli M, Savino S, Fontana MR, et al. 2005. Ng-MIP, a surface-exposed lipoprotein of *Neisseria gonorrhoeae*, has a peptidyl-prolyl cis/trans isomerase (PPlase) activity and is involved in persistence in macrophages. *Mol. Microbiol.* 58(3):669–81

106. Liu Y, Egilmez NK, Russell MW. 2013. Enhancement of adaptive immunity to *Neisseria gonorrhoeae* by local intravaginal administration of microencapsulated interleukin 12. *J. Infect. Dis.* 208(11):1821–29

107. Liu Y, Feinen B, Russell MW. 2011. New concepts in immunity to *Neisseria gonorrhoeae*: innate responses and suppression of adaptive immunity favor the pathogen, not the host. *Front. Microbiol.* 2(MAR):52

108. Liu Y, Hammer LA, Liu W, Hobbs MM, Zielke RA, et al. 2017. Experimental vaccine induces Th1-driven immune responses and resistance to *Neisseria gonorrhoeae* infection in a murine model. *Mucosal Immunol.* 10(6):1594–1608

109. Liu Y, Islam EA, Jarvis GA, Gray-Owen SD, Russell MW. 2012. *Neisseria gonorrhoeae* selectively suppresses the development of Th1 and Th2 cells, and enhances Th17 cell responses, through TGF-β-dependent mechanisms. *Mucosal Immunol.* 5(3):320–31

110. Liu Y, Liu W, Russell MW. 2014. Suppression of host adaptive immune responses by *Neisseria gonorrhoeae*: role of interleukin 10 and type 1 regulatory T cells. *Mucosal Immunol.* 7(1):165–76

111. Liu Y, Perez J, Hammer LA, Gallagher HC, De Jesus M, et al. 2018. Intravaginal administration of interleukin 12 during genital gonococcal infection in mice

induces immunity to heterologous strains of *Neisseria gonorrhoeae*. *mSphere*. 3(1): e00421-17

112. Liu Y, Russell MW. 2011. Diversion of the immune response to *Neisseria gonorrhoeae* from Th17 to Th1/Th2 by treatment with anti-transforming growth factor β antibody generates immunological memory and protective immunity. *MBio*. 2(3): e00095-11

113. Lovett A, Duncan JA. 2019. Human immune response and the natural history of *Neisseria gonorrhoeae* infection. *Front. Immunol.* 10(FEB):1–10

114. Maisey K, Nardocci G, Imarai M, Cardenas H, Rios M, et al. 2003. Expression of proinflammatory cytokines and receptors by human fallopian tubes in organ culture following challenge with *Neisseria gonorrhoeae*. *Infect. Immun.* 71(1):527–32

115. Masson L, Mlisana K, Little F, Werner L, Mkhize NN, et al. 2014. Defining genital tract cytokine signatures of sexually transmitted infections and bacterial vaginosis in women at high risk of HIV infection: A cross-sectional study. *Sex. Transm. Infect.* 90(8):580–87

116. McCormack WM, Reynolds GH. 1982. Effect of menstrual cycle and method of contraception on recovery of *Neisseria gonorrhoeae*. *JAMA*. 247(9):1292–94

117. McGee ZA, Clemens CM, Jensen RL, Klein JJ, Barley LR, Gorby GL. 1992. Local induction of tumor necrosis factor as a molecular mechanism of mucosal damage by gonococci. *Microb. Pathog.* 12(5):333–41

118. McGowin CL, Spagnuolo RA, Pyles RB. 2010. *Mycoplasma genitalium* rapidly disseminates to the upper reproductive tracts and knees of female mice following vaginal inoculation. *Infect. Immun.* 78(2):726–36

119. McLaughlin SE, Ghanem KG, Zenilman JM, Griffiss JML. 2019. Risk of gonococcal infection during vaginal exposure is associated with high vaginal pH and active menstruation. *Sex. Transm. Dis.* 46(2):86–90

120. McLean AC, Valenzuela N, Fai S, Bennett SAL. 2012. Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrous cycle staging identification. *J. Vis. Exp.*, p. 4389

121. Merz AJ, So M. 2000. Interactions of pathogenic Neisseriae with epithelial cell membranes. *Annu. Rev. Cell Dev. Biol.* 16:423–57

122. Meyn LA, Macio I, Darville T, Wiesenfeld HC, Hillier SL. 2016. Microbiological etiology of symptomatic versus subclinical pelvic inflammatory disease. *Am. J. Obstet. Gynecol.* 215(6):S816

123. Miller WC, Ford CA, Morris M, Handcock MS, Schmitz JL, et al. 2004.

Prevalence of chlamydial and gonococcal infections among young adults in the United States. *J. Am. Med. Assoc.* 291(18):2229–36

124. Mitchell C, Prabhu M. 2013. Pelvic inflammatory disease: Current concepts in pathogenesis, diagnosis and treatment. *Infect. Dis. Clin. North Am.* 27(4):793–809

125. Miyairi I, Ramsey KH, Patton DL. 2010. Duration of untreated chlamydial genital infection and factors associated with clearance: Review of animal studies. *J. Infect. Dis.* 201(S2):96–103

126. Moller BR, Taylor-Robinson D, Purr PM. 1984. Serological evidence implicating *Mycoplasma genitalium* in pelvic inflammatory disease. *Lancet*. 323(8386):1102–3

127. Morrison RP, Caldwell HD. 2002. Immunity to murine chlamydial genital infection. *Infect. Immun.* 70(6):2741–51

128. Morrison RP, Feilzer K, Tumas DB. 1995. Gene knockout mice establish a primary protective role for major histocompatibility complex class II-restricted responses in *Chlamydia trachomatis* genital tract infection. *Infect. Immun.* 63(12):4661–68

129. Morrison SG, Su H, Caldwell HD, Morrison RP. 2000. Immunity to murine *Chlamydia trachomatis* genital tract reinfection involves B cells and CD4+ T cells but not CD8+ T cells. *Infect. Immun.* 68(12):6979–87

130. Mount DT, Bigazzi PE, Barron AL. 1973. Experimental genital infection of male guinea pigs with the agent of guinea pig inclusion conjunctivitis and transmission to females. *Infect. Immun.* 8(6):925–30

131. Murthy AK, Li W, Chaganty BKR, Kamalakaran S, Guentzel MN, et al. 2011. Tumor necrosis factor alpha production from CD8 + T cells mediates oviduct pathological sequelae following primary genital *Chlamydia muridarum* infection. *Infect. Immun.* 79(7):2928–35

132. Murthy AK, Li W, Ramsey KH. 2016. Immunopathogenesis of chlamydial infections. *Curr. Top. Microbiol. Immunol.* 412:183–215

133. Ness RB, Hillier SL, Kip KE, Soper DE, Stamm CA, et al. 2004. Bacterial vaginosis and risk of pelvic inflammatory disease. *Obstet. Gynecol.* 104(4):761–69

134. Ness RB, Keder LM, Soper DE, Amortegui AJ, Gluck J, et al. 1997. Oral contraception and the recognition of endometritis. *Am. J. Obstet. Gynecol.* 176(3):580–85

135. Ness RB, Kip KE, Soper DE, Hillier S, Stamm CA, et al. 2005. Bacterial vaginosis (BV) and the risk of incident gonococcal or chlamydial genital infection in a predominantly black population. *Sex. Transm. Dis.* 32(7):413–17

136. Ness RB, Soper DE, Holley RL, Peipert J, Randall H, et al. 2001. Hormonal and barrier contraception and risk of upper genital tract disease in the PID Evaluation and Clinical Health (PEACH) study. *Am. J. Obstet. Gynecol.* 185(1):121–27

137. Ness RB, Trautmann G, Richter HE, Randall H, Peipert JF, et al. 2005. Effectiveness of treatment strategies of some women with pelvic inflammatory disease: a randomized trial. *Obstet. Gynecol.* 106(3):573–80

138. Neumann W, Hadley RC, Nolan EM. 2017. Transition metals at the host-pathogen interface: How *Neisseria* exploit human metalloproteins for acquiring iron and zinc. *Essays Biochem.* 61(2):211–23

139. Newhall WJ, Batteiger B, Jones RB. 1982. Analysis of the human serological response to proteins of *Chlamydia trachomatis*. *Infect. Immun.* 38(3):1181–89

140. Newman L, Rowley J, Hoorn S Vander, Wijesooriya NS, Unemo M, et al. 2015. Global estimates of the prevalence and incidence of four curable sexually transmitted infections in 2012 based on systematic review and global reporting. *PLoS One*. 10(12):e0143304

141. Ngampasutadol J, Tran C, Gulati S, Blom AM, Jerse AE, et al. 2008. Species-specificity of *Neisseria gonorrhoeae* infection: Do human complement regulators contribute? *Vaccine*. 26(SUPPL. 8):I62-6

142. Nsuami M, Cammarata CL, Brooks BN, Taylor SN, Martin DH. 2004. Chlamydia and gonorrhea co-occurrence in a high school population. *Sex. Transm. Dis.* 31(7):424–27

143. Nunes A, Gomes JP. 2014. Evolution, phylogeny, and molecular epidemiology of Chlamydia. *Infect. Genet. Evol.* 23:49–64

144. O'Connell CM, Ferone ME. 2016. *Chlamydia trachomatis* genital infections. *Microb. cell (Graz, Austria)*. 3(9):390–403

145. Oftung F, Lovik M, Andersen SR, Froholm LO, Bjune G. 1999. A mouse model utilising human transferrin to study protection against *Neisseria meningitidis* serogroup B induced by outer membrane vesicle vaccination. *FEMS Immunol. Med. Microbiol.* 26(1):75–82

146. Ortiz MC, Lefimil C, Rodas PI, Vernal R, Lopez M, et al. 2015. *Neisseria gonorrhoeae* modulates immunity by polarizing human macrophages to a M2 profile. *PLoS One*. 10(6):e0130713

147. Packiam M, Veit SJ, Anderson DJ, Ingalls RR, Jerse AE. 2010. Mouse strain-dependent differences in susceptibility to *Neisseria gonorrhoeae* infection and induction of innate immune responses. *Infect. Immun.* 78(1):433–40

148. Palmer A, Criss AK. 2018. Gonococcal defenses against antimicrobial activities of

neutrophils. *Trends Microbiol.* 26(12):1022–34

149. Pasquale SA. 1989. Noncontraceptive health benefits and risks of steroidal contraception. *Int. J. Fertil.* 34 Suppl:85–87

150. Pattanasin S, Dunne EF, Wasinrapee P, Tongtoyai J, Chonwattana W, et al. 2018. Screening for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infection among asymptomatic men who have sex with men in Bangkok, Thailand. *Int. J. STD AIDS*. 29(6):577–87

151. Patton DL, Halbert SA, Kuo CC, Wang SP, Holmes KK. 1983. Host response to primary *Chlamydia trachomatis* infection of the fallopian tube in pig-tailed monkeys. *Fertil. Steril.* 40(6):829–40

152. Patton DL, Kuo C-C, Wang S-P, Brenner RM, Sternfeld MD, et al. 1987. Chlamydial infection of subcutaneous fimbrial transplants in cynomolgus and rhesus monkeys. *J. Infect. Dis.* 155(2):229–35

153. Patton DL, Sweeney YTC, Kuo CC. 1994. Oral contraceptives do not alter the course of experimentally induced chlamydial salpingitis in monkeys. *Sex. Transm. Dis.* 21(2):89–92

154. Petousis-Harris H, Paynter J, Morgan J, Saxton P, McArdle B, et al. 2017. Effectiveness of a group B outer membrane vesicle meningococcal vaccine against gonorrhoea in New Zealand: a retrospective case-control study. *Lancet*. 390(10102):1603–10

155. Pilligua-Lucas M, Prudhomme I, Costenoble-Caherty A, Jerse A. 2016. Establishment of *Neisseria gonorrhoeae* upper reproductive tract infection in female mice through the use of human transferrin supplementation for improved models of gonorrhea and gonorrhea/chlamydia coinfection. Poster presented at 20th International Pathogenic Neisseria Conference, 4th-9th September 2016. Manchester, United Kingdom

156. Pioli PA, Amiel E, Schaefer TM, Connolly JE, Wira CR, Guyre PM. 2004. Differential expression of toll-like receptors 2 and 4 in tissues of the human female reproductive tract. *Infect. Immun.* 72(10):5799–5806

157. Quillin SJ, Seifert HS. 2018. *Neisseria gonorrhoeae* host adaptation and pathogenesis. *Nat. Rev. Microbiol.* 16(4):226–40

158. Ram S, Mackinnon FG, Gulati S, McQuillen DP, Vogel U, et al. 1999. The contrasting mechanisms of serum resistance of *Neisseria gonorrhoeae* and group B *Neisseria meningitidis*. *Mol. Immunol.* 36(13–14):915–28

159. Rank RG, Lacy HM, Goodwin A, Sikes J, Whittimore J, et al. 2010. Host chemokine and cytokine response in the endocervix within the first developmental cycle of *Chlamydia muridarum*. *Infect. Immun.* 78(1):536–44

160. Rank RG, Sanders MM. 1992. Pathogenesis of endometritis and salpingitis in a guinea pig model of chlamydial genital infection. *Am. J. Pathol.* 140(4):927–36

161. Rank RG, Sanders MM, Kidd AT. 1993. Influence of the estrous cycle on the development of upper genital tract pathology as a result of chlamydial infection in the guinea pig model of pelvic inflammatory disease. *Am. J. Pathol.* 142(4):1291–96

162. Rank RG, White HJ, Hough AJ, Pasley JN, Barron AL. 1982. Effect of estradiol on chlamydial genital infection of female guinea pigs. *Infect. Immun.* 38(2):699–705

163. Rank RG, Whittimore J, Bowlin AK, Dessus-Babus S, Wyrick PB. 2008. Chlamydiae and polymorphonuclear leukocytes: unlikely allies in the spread of chlamydial infection. *FEMS Immunol. Med. Microbiol.* 54(1):104–13

164. Rank RG, Whittum-Hudson JA. 2010. Protective immunity to chlamydial genital infection: Evidence from animal studies. *J. Infect. Dis.* 201(SUPPL. 2):S168-77

165. Raterman EL, Jerse AE. 2019. Female mouse model of *Neisseria gonorrhoeae infection*. In *Methods in Molecular Biology*. 1997:413–29. Humana Press Inc.

166. Reekie J, Donovan B, Guy R, Hocking J, Jorm L, et al. 2013. Comparison of the rate of hospitalisation For pelvic inflammatory disease (PID) following a diagnosis of chlamydia or gonorrhoea in women resident in New South Wales, Australia. *Sex. Transm. Infect.* 89(Suppl 1):A150.3-A151

167. Reekie J, Donovan B, Guy R, Hocking JS, Jorm L, et al. 2014. Hospitalisations for pelvic inflammatory disease temporally related to a diagnosis of chlamydia or gonorrhoea: A retrospective cohort study. *PLoS One*. 9(4):e94361

168. Riley MM, Zurenski MA, Frazer LC, O'Connell CM, Andrews CW, et al. 2012. The recall response induced by genital challenge with *Chlamydia muridarum* protects the oviduct from pathology but not from reinfection. *Infect. Immun.* 80(6):2194–2203

169. Roshick C, Wood H, Caldwell HD, McClarty G. 2006. Comparison of gamma interferon-mediated antichlamydial defense mechanisms in human and mouse cells. *Infect. Immun.* 74(1):225–38

170. Rotman E, Seifert HS. 2014. The genetics of Neisseria species. *Annu. Rev. Genet.* 48(1):405–31

171. Rouquette-Loughlin CE, Zalucki YM, Dhulipala VL, Balthazar JT, Doyle RG, et al. 2017. Control of *gdhR* expression in *Neisseria gonorrhoeae* via autoregulation and a master repressor (MtrR) of a drug efflux pump operon. *MBio*. 8(2):e00449-17

172. Russell MW, Jerse AE, Gray-Owen SD. 2019. Progress toward a gonococcal vaccine: the way forward. *Front. Immunol.* 10:2417

173. Sanchez L, Calvo M, Brock JH. 1992. Biological role of lactoferrin. *Arch. Dis. Child.* 67(5):657–61

174. Satterwhite CL, Torrone E, Meites E, Dunne EF, Mahajan R, et al. 2013. Sexually transmitted infections among US women and men: Prevalence and incidence estimates, 2008. *Sex. Transm. Dis.* 40(3):187–93

175. Seifert HS. 1996. Questions about gonococcal pilus phase- and antigenic variation. *Mol. Microbiol.* 21(3):433–40

176. Seifert HS. 2019. Location, location, location—commensalism, damage and evolution of the pathogenic Neisseria. *J. Mol. Biol.* 431(16):3010–14

177. Shah AA, Schripsema JH, Imtiaz MT, Sigar IM, Kasimos J, et al. 2005. Histopathologic changes related to fibrotic oviduct occlusion after genital tract infection of mice with *Chlamydia muridarum*. *Sex. Transm. Dis.* 32(1):49–56

178. Sheldon IM, Owens SE, Turner ML. 2017. Innate immunity and the sensing of infection, damage and danger in the female genital tract. *J. Reprod. Immunol.* 119:67–73

179. Shen M, Senthil Kumar SPD, Shi H. 2014. Estradiol regulates insulin signaling and inflammation in adipose tissue. *Horm. Mol. Biol. Clin. Investig.* 17(2):99–107

180. Short VL, Totten PA, Ness RB, Astete SG, Kelsey SF, Haggerty CL. 2009. Clinical presentation of *Mycoplasma genitalium* infection versus Neisseria gonorrhoeae infection among women with pelvic inflammatory disease. *Clin. Infect. Dis.* 48(1):41–47

181. Shrestha S. 2011. Influence of host genetic and ecological factors in complex concomitant infections - relevance to sexually transmitted infections. *J. Reprod. Immunol.* 92(1–2):27–32

182. Simms I, Eastick K, Mallinson H, Thomas K, Gokhale R, et al. 2003. Associations between *Mycoplasma genitalium*, *Chlamydia trachomatis*, and pelvic inflammatory disease. *Sex. Transm. Infect.* 79(2):154–56

183. Simms I, Stephenson JM, Mallinson H, Peeling RW, Thomas K, et al. 2006. Risk factors associated with pelvic inflammatory disease. *Sex. Transm. Infect.* 82(6):452–57

184. Sitruk-Ware R, Nath A, Mishell DR. 2013. Contraception technology: Past, present and future. *Contraception*. 87(3):319–30

185. Soboll G, Schaefer TM, Wira CR. 2006. Effect of Toll-like receptor (TLR)

agonists on TLR and microbicide expression in uterine and vaginal tissues of the mouse. *Am. J. Reprod. Immunol.* 55(6):434–46

186. Song CH, Kim N, Sohn SH, Lee SM, Nam RH, et al. 2018. Effects of 17β-estradiol on colonic permeability and inflammation in an azoxymethane/dextran sulfate sodium-induced colitis mouse model. *Gut Liver*. 12(6):682–93

187. Song W, Condron S, Mocca BT, Veit SJ, Hill D, et al. 2008. Local and humoral immune responses against primary and repeat *Neisseria gonorrhoeae* genital tract infections of 17β-estradiol-treated mice. *Vaccine*. 26(45):5741–51

188. Spencer SE, Valentin-Bon IE, Whaley K, Jerse AE. 2004. Inhibition of *Neisseria gonorrhoeae* genital tract infection by leading-candidate topical microbicides in a mouse model. *J. Infect. Dis.* 189(3):410–19

189. Spinillo A, Gorini G, Piazzi G, Baltaro F, Monaco A, Zara F. 1996. The impact of oral contraception on chlamydial infection among patients with pelvic inflammatory disease. *Contraception*. 54(3):163–68

190. Stojijkovic I, Hwa V, de Saint Martin L, O'Gaora P, Nassif X, et al. 1995. The *Neisseria meningitidis* haemoglobin receptor: its role in iron utilization and virulence. *Mol. Microbiol.* 15(3):531–41

191. Stork M, Grijpstra J, Bos MP, Mañas Torres C, Devos N, et al. 2013. Zinc piracy as a mechanism of *Neisseria meningitidis* for evasion of nutritional immunity. *PLoS Pathog.* 9(10):e1003733

192. Strange HR, Zola TA, Cornelissen CN. 2011. The *fbpABC* operon is required for ton-independent utilization of xenosiderophores by *Neisseria gonorrhoeae* strain FA19. *Infect. Immun.* 79(1):267–78

193. Stupiansky NW, Van Der Pol B, Williams JA, Weaver B, Taylor SE, Fortenberry JD. 2011. The natural history of incident gonococcal infection in adolescent women. *Sex. Transm. Dis.* 38(8):750–54

194. Sun X, Tian Q, Wang L, Xue M, Zhong G. 2017. IL-6-mediated signaling pathways limit *Chlamydia muridarum* infection and exacerbate its pathogenicity in the mouse genital tract. *Microbes Infect.* 19(11):536–45

195. Sutton MY, Sternberg M, Zaidi A, St. Louis ME, Markowitz LE. 2005. Trends in pelvic inflammatory disease hospital discharges and ambulatory visits, United States, 1985-2001. *Sex. Transm. Dis.* 32(12):778–84

196. Sweet RL. 2011. Treatment of acute pelvic inflammatory disease. *Infect. Dis. Obstet. Gynecol.* 2011:561909

197. Tabibzadeh S, Sun XZ. 1992. Cytokine expression in human endometrium throughout the menstrual cycle. *Hum. Reprod.* 7(9):1214–21

198. Takebayashi A, Kimura F, Kishi Y, Ishida M, Takahashi A, et al. 2014. The association between endometriosis and chronic endometritis. *PLoS One*. 9(2):e88354

199. Takeuchi T, Yoshida M, Shimizu T, Asano A, Shimokawa T, et al. 2013. Differential expressions of toll-like receptor genes in the vagina of pregnant mice. *J. Vet. Med. Sci.* 75(5):561–65

200. Taylor BD, Darville T, Ferrell RE, Ness RB, Haggerty CL. 2013. Racial variation in toll-like receptor variants among women with pelvic inflammatory disease. *J. Infect. Dis.* 207(6):940–46

201. Taylor BD, Darville T, Haggerty CL. 2013. Does bacterial vaginosis cause pelvic inflammatory disease? *Sex Transm Dis*. 40(2):117-22

202. Taylor BD, Ness RB, Darville T, Haggerty CL. 2011. Microbial correlates of delayed care for pelvic inflammatory disease. *Sex. Transm. Dis.* 38(5):434–38

203. Timmerman MM, Shao JQ, Apicella MA. 2005. Ultrastructural analysis of the pathogenesis of *Neisseria gonorrhoeae* endometrial infection. *Cell. Microbiol.* 7(5):627–36

204. Tongtoyai J, Todd CS, Chonwattana W, Pattanasin S, Chaikummao S, et al. 2015. Prevalence and correlates of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* by anatomic site among urban Thai men who have sex with men. *Sex. Transm. Dis.* 42(8):440–49

205. Tuffrey M, Taylor-Robinson D. 1981. Progesterone as a key factor in the development of a mouse model for genital-tract infection with *Chlamydia trachomatis*. *FEMS Microbiol. Lett.* 12(2):111–15

206. Unemo M. 2015. Current and future antimicrobial treatment of gonorrhoea - the rapidly evolving *Neisseria gonorrhoeae* continues to challenge. *BMC Infect Dis*. 2015 Aug 21;15:364

207. Valdivia RH. 2008. Chlamydia effector proteins and new insights into chlamydial cellular microbiology. *Curr. Opin. Microbiol.* 11(1):53–59

208. Veale DR, Goldner M, Penn CW, Ward J, Smith H. 1979. The intracellular survival and growth of gonococci in human phagocytes. *J. Gen. Microbiol.* 113(2):383–93

209. Von Wolff M, Thaler CJ, Strowitzki T, Broome J, Stolz W, Tabibzadeh S. 2000. Regulated expression of cytokines in human endometrium throughout the menstrual cycle: Dysregulation in habitual abortion. *Mol. Hum. Reprod.* 6(7):627–34

210. Vonck RA, Darville T, O'Connell CM, Jerse AE. 2011. Chlamydial infection

increases gonococcal colonization in a novel murine coinfection model. *Infect. Immun.* 79(4):1566–77

211. Walker C, Landers D, Ohm-Smith M, Robbie M, Luft J, et al. 1991. Comparison of cefotetan plus doxycycline with cefoxitin plus doxycycline in the inpatient treatment of acute salpingitis. *Sex. Transm. Dis.* 18(2):119–23

212. Wally J, Buchanan SK. 2007. A structural comparison of human serum transferrin and human lactoferrin. *BioMetals*. 20(3–4):249–62

213. Welter-Stahl L, Ojcius DM, Viala J, Girardin S, Liu W, et al. 2006. Stimulation of the cytosolic receptor for peptidoglycan, Nod1, by infection with *Chlamydia trachomatis* or *Chlamydia muridarum*. *Cell. Microbiol.* 8(6):1047–57

214. Weston EJ, Workowski K, Torrone E, Weinstock H, Stenger MR. 2018. Adherence to CDC recommendations for the treatment of uncomplicated gonorrhea — STD surveillance network, United States, 2016. *MMWR. Morb. Mortal. Wkly. Rep.* 67(16):473–76

215. Weström L. 1975. Effect of acute pelvic inflammatory disease on fertility. *Am. J. Obstet. Gynecol.* 121(5):707–13

216. Weström L, Joesoef R, Reynolds G, Hagdu A, Thompson SE. 1992. Pelvic inflammatory disease and fertility. A cohort study of 1,844 women with laparoscopically verified disease and 657 control women with normal laparoscopic results. *Sex. Transm. Dis.* 19(4):185–92

217. Wiesenfeld HC, Hillier SL, Krohn MA, Amortegui AJ, Heine RP, et al. 2002. Lower genital tract infection and endometritis: Insight into subclinical pelvic inflammatory disease. *Obstet. Gynecol.* 100(3):456–63

218. Wiesenfeld HC, Hillier SL, Meyn LA, Amortegui AJ, Sweet RL. 2012. Subclinical pelvic inflammatory disease and infertility. *Obstet. Gynecol.* 120(1):37–43

219. Wiesenfeld HC, Manhart LE. 2017. *Mycoplasma genitalium* in women: Current knowledge and research priorities for this recently emerged pathogen. *J. Infect. Dis.* 216(suppl_2):S389–95

220. Wiesenfeld HC, Sweet RL, Ness RB, Krohn MA, Amortegui AJ, Hillier SL. 2005. Comparison of acute and subclinical pelvic inflammatory disease. *Sex. Transm. Dis.* 32(7):400–405

221. Wira CR, Fahey J V., Ghosh M, Patel M V., Hickey DK, Ochiel DO. 2010. Sex hormone regulation of innate immunity in the female reproductive tract: the role of epithelial cells in balancing reproductive potential with protection against sexually transmitted pathogens. *Am. J. Reprod. Immunol.* 63(6):544–65

222. Wira CR, Fahey J V., Rodriguez-Garcia M, Shen Z, Patel M V. 2014. Regulation

of mucosal immunity in the female reproductive tract: The role of sex hormones in immune protection against sexually transmitted pathogens. *Am. J. Reprod. Immunol.* 72(2):236–58

223. Wira CR, Fahey J V., Sentman CL, Pioli PA, Shen L. 2005. Innate and adaptive immunity in female genital tract: Cellular responses and interactions. *Immunol. Rev.* 206:306–35

224. Wira CR, Grant-Tschudy KS, Crane-Godreau MA. 2005. Epithelial cells in the female reproductive tract: a central role as sentinels of immune protection. *Am. J. Reprod. Immunol.* 53(2):65–76

225. Wira CR, Rodriguez-Garcia M, Patel M V. 2015. The role of sex hormones in immune protection of the female reproductive tract. *Nat. Rev. Immunol.* 15(4):217–30

226. Wira CR, Rossoll RM. 1995. Antigen-presenting cells in the female reproductive tract: influence of sex hormones on antigen presentation in the vagina. *Immunology*. 84(4):505–8

227. Wolfgang M, Van Putten JPM, Hayes SF, Koomey M. 1999. The *comP* locus of *Neisseria gonorrhoeae* encodes a type IV prepilin that is dispensable for pilus biogenesis but essential for natural transformation. *Mol. Microbiol.* 31(5):1345–57

228. Wølner-Hanssen P, Eschenbach DA, Paavonen J, Kiviat N, Stevens CE, et al. 1990. Decreased risk of symptomatic chlamydial pelvic inflammatory disease associated with oral contraceptive use. *JAMA*. 263(1):54–59

229. Workowski KA, Bolan GA. 2015. Sexually transmitted diseases treatment guidelines, 2015. *MMWR Recomm. Reports*. 64(3):1–138

230. Yang L-G, Zhang X-H, Zhao P-Z, Chen Z-Y, Ke W-J, et al. 2018. Gonorrhea and chlamydia prevalence in different anatomical sites among men who have sex with men: a cross-sectional study in Guangzhou, China. *BMC Infect. Dis.* 18(1):675

231. Zarantonelli ML, Szatanik M, Giorgini D, Hong E, Huerre M, et al. 2007. Transgenic mice expressing human transferrin as a model for meningococcal infection. *Infect. Immun.* 75(12):5609–14

232. Zervomanolakis I, Ott HW, Hadziomerovic D, Mattle V, Seeber BE, et al. 2007. Physiology of upward transport in the human female genital tract. *Ann. N. Y. Acad. Sci.* 1101:1–20

233. Zhang X, Starnbach MN. 2015. An excess of the proinflammatory cytokines IFN-γ and IL-12 Impairs the development of the memory CD8 + T cell response to *Chlamydia trachomatis*. *J. Immunol.* 195(4):1665–75

234. Zhou X, Gao X, Broglie PM, Kebaier C, Anderson JE, et al. 2014. Hexa-acylated

lipid A is required for host inflammatory response to *Neisseria gonorrhoeae* in experimental gonorrhea. *Infect. Immun.* 82(1):184–92

235. Zhu W, Hunt DJ, Richardson AR, Stojiljkovic I. 2000. Use of heme compounds as iron sources by pathogenic neisseriae requires the product of the *hemO* gene. *J. Bacteriol.* 182(2):439–47

236. Zielke RA, Le Van A, Baarda BI, Herrera MF, Acosta CJ, et al. 2018. SliC is a surface-displayed lipoprotein that is required for the anti-lysozyme strategy during *Neisseria gonorrhoeae* infection. *PLoS Pathog.* 14(7): e1007081

237. Zughaier SM, Kandler JL, Shafer WM. 2014. *Neisseria gonorrhoeae* modulates iron-limiting innate immune defenses in macrophages. *PLoS One*. 9(1):e87688

238. Zygiel EM, Nolan EM. 2018. Transition metal sequestration by the host-defense protein calprotectin. *Annu. Rev. Biochem.* 87(1):621–43