Chlamydial Infection Increases Gonococcal Colonization in a Novel Murine Coinfection Model

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Abstract

Genital tract infections caused by *Neisseria gonorrhoeae* and *Chlamydia trachomatis* serovars D-K occur at high incidence in many areas of the world. Despite high rates of coinfection with these pathogens, investigations of host-parasite interactions have focused on each pathogen individually. Here we describe a coinfection model in which female BALB/c mice were first infected with the mouse *Chlamydia* species *C. muridarum* and then inoculated with *N. gonorrhoeae* following treatment with water-soluble 17β-estradiol to promote long-term gonococcal infection. Viable gonococci and chlamydiae were recovered for an average of 8-10 days, and diplococci and chlamydial inclusions were observed in lower genital tract tissue by immunohistochemical staining. Estradiol treatment reduced proinflammatory cytokine and chemokine levels in chlamydia-infected mice; however, coinfected mice had a higher percentage of vaginal neutrophils compared to mice infected with either pathogen alone. We detected no difference in pathogen-specific antibody levels due to coinfection. Interestingly, significantly more gonococci were recovered from coinfected mice compared to mice infected with *N. gonorrhoeae* alone. We found no evidence that *C. muridarum* increases gonococcal adherence to, or invasion of immortalized murine epithelial cells. However, increased vaginal concentrations of inflammatory mediators MIP-2 and TNF-α were detected in *C. muridarum*-infected mice prior to inoculation with *N. gonorrhoeae* concurrently with down-regulation of cathelicidin-related antimicrobial peptide (CRAMP) and secretory leukocyte peptidase inhibitor (SLPI) genes. We conclude that female mice can be successfully infected with both *C. muridarum* and *N. gonorrhoeae*, and that chlamydia-induced alterations in host innate responses may enhance gonococcal infection.
Chlamydia and gonorrhea are the two most common notifiable infectious diseases in the United States with over 1 million cases of chlamydia and 350,000 cases of gonorrhea reported to the Centers for Disease Control in 2008 (9). Actual rates of infection are much higher due to high rates of asymptomatic infection (50). As many as 50-70% of individuals with gonorrhea also have a chlamydial infection (20, 50, 55), and empirical treatment for chlamydia upon detection of *N. gonorrhoeae* is recommended (10, 27). *Neisseria gonorrhoeae* and *Chlamydia trachomatis* are both Gram-negative, human-specific pathogens. In symptomatic infections, both organisms elicit a proinflammatory response characterized by the influx of polymorphonuclear leukocytes (PMNs). Clinically, gonorrhea is typically more pyogenic. Post-infection complications can occur with either pathogen and complications are generally more common and more severe in women. Infections that ascend to the upper genital tract in women lead to pelvic inflammatory disease (PID), the complications of which include chronic pelvic pain, ectopic pregnancy, and infertility (28, 78).

The incidence (50, 55), transmission (45, 46, 48), and associated symptoms and complications (67, 68) of gonococcal and chlamydial coinfection have been examined in epidemiological studies. However, differences in the pathogenesis and host response to coinfection have not been investigated in an infection model. *N. gonorrhoeae* is primarily extracellular, but can invade and replicate within epithelial cells (19, 23). While the inflammatory response to *N. gonorrhoeae* can be robust in symptomatic infections, gonococcal infection induces only a transient antibody response (76). Recent evidence suggests that IL-17 responses are induced during gonococcal infection and that both IL17 (25) and toll-like receptor 4 (TLR4) are protective (59). In contrast, *C. trachomatis* is an obligate, intracellular parasite. 


that undergoes a complex life cycle within the host cell involving the infectious, metabolically
inactive elementary body (EB) and the metabolically active, replicative reticulate body (RB) (1).
The primary immune response to *C. trachomatis* is through the Th1 pathway (16).

Despite these differences in life-styles and host response, how one organism may alter the
pathogenesis, disease severity, susceptibility and host response to the other pathogen is not
known. A small animal model of gonococcal and chlamydial coinfection is needed to facilitate
the investigation of aspects of pathogenesis that are unique to coinfection and the development of
improved prophylactic products. Well-established female mouse models of gonococcal or
chlamydial infection currently exist. The development of a coinfection model, however, is
challenged by differences in the susceptibility of mice to each pathogen with respect to the stage
of the reproductive cycle. The mouse model of gonococcal genital tract infection capitalizes on
the transient susceptibility of female mice to *N. gonorrhoeae* that occurs during the proestrus
stage of the estrous cycle (15, 35). In this model, mice are treated with 17β-estradiol and
antibiotics to promote long-term colonization with *N. gonorrhoeae* (32). In the most recent
modification of this model, water-soluble estradiol is used, and serum estradiol concentrations
return to physiological levels by day 3 post-infection and mice are colonized for 10-12 days (76).
Gonococci are detected within murine vaginal and cervical tissue (76) and ascend to the upper
genital tract in approximately 18-20% of mice (32). Infected mice have an inflammatory
response that is characterized by PMN influx and the induction of TNFα, IL-6, MIP-2, and KC
(32, 58, 75), and similar to human infection, mice elicit an unremarkable humoral response, and
are susceptible to repeat infection with the same strain (76). Female mice are most susceptible to
*Chlamydia* species when in the progesterone-dominant phase of the reproductive cycle and
protocols for infection with *Chlamydia muridarum* (formerly *C. trachomatis* MoPn) (3) or
human serovars of *C. trachomatis* that use or do not use progesterone treatment have been developed (3, 6, 62). The course of infection with the mouse pneumonitis agent, *C. muridarum*, appears more similar to human genital chlamydial disease than infection of mice with human serovars of *C. trachomatis*. *C. muridarum* ascends from the lower to the upper genital tract of female mice (3) and causes an inflammatory response that is characterized by infiltration of both acute and chronic inflammatory cells, and post-infection sequelae, such as tubal occlusion, hydrosalpinx, and infertility (16, 18).

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

Bacterial propagation. *N. gonorrhoeae* strain FA1090 [porB1b, AHU (an auxotype for arginine, hypoxanthine, and uracil), serum resistant] was originally isolated from a female with disseminated gonococcal infection (12). Frozen stocks of piliated, OpaB-expressing FA1090 bacteria isolated from a male volunteer (33) were passaged on solid GC agar containing Kellogg’s supplement I (37) and 12 µM Fe(NO$_3$)$_3$ and incubated at 37°C in a humidified 7% CO$_2$ incubator. GC agar with antibiotic selection [GC-vancomycin, colistin, nystatin, trimethoprim sulfate, and streptomycin (VCNTS)] and heart infusion agar (HIA) were used to isolate *N. gonorrhoeae* and facultatively anaerobic commensal flora, respectively, from murine vaginal mucus as described (32). *C. muridarum* strain Nigg (70) was propagated in L929 mouse fibroblast cells (gift of Dr. Anthony T. Maurelli, Uniformed Services University, Bethesda, MD) and *C. trachomatis* serovar D (gift of Dr. Anthony T. Maurelli) was propagated in ME180 human cervical epithelial cells, similarly to the method described by O’Connell and Nicks (57). Briefly, monolayers of L929 or ME180 cells in 24-well plates were inoculated with *C. muridarum* or *C. trachomatis*, respectively, suspended in infection media [1X Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 µg/ml gentamicin, and 100 µg/ml cycloheximide] at a multiplicity of infection (MOI) of 1. Plates were centrifuged at 1600 x g for 1 hr at 37°C and media was replaced with fresh infection media. Infected monolayers were incubated at 37°C, 5% CO$_2$ for 36 hrs before being harvested into SPG buffer (218 mM sucrose, 3.8 mM KH$_2$PO$_4$, 7.2 mM K$_2$HPO$_4$, 4.9 mM L-glutamate, pH 7.2), sonicated briefly, and frozen at -80°C. The titer of inclusion forming units (IFU) in each stock was determined by immunofluorescence as described by Kelly *et al.* (39) except that inclusions were stained with ChlamIII anti-chlamydial LPS antibody (Santa Cruz Biotechnology) at a 1:500
dilution and goat anti-mouse AlexaFlour-488 secondary antibody (Invitrogen) at a 1:2000 dilution. L929 mouse fibroblast cells were maintained in DMEM with 10% FBS and grown to large quantities in suspension culture in RPMI with 5% FBS. ME180 human cervical epithelial cells were maintained in McCoy’s 5A media supplemented with 10% FBS and 2.2 g/L NaHCO₃. Solid agar, cell culture reagents, and chemicals were purchased from Difco, Quality Biological, and Sigma, respectively, unless otherwise noted.

**Coinfection protocol.** Female BALB/c mice 4-6 weeks in age were purchased from the National Cancer Institute (Bethesda, MD). The infection protocol is shown in Figure 1. Except when noted, each experiment consisted of four groups of mice: mice coinfected with *N. gonorrhoeae* and *C. muridarum*, mice infected with either pathogen alone, and mice inoculated with buffers alone as a control for inflammation (n = 10-11 mice per group in each of three experiments). Inoculations with *C. muridarum* were preformed on anesthetized mice for the purpose of immobilization by intraperitoneal (i.p.) injection of a ketamine/xylazine mixture (10 mg and 1.5 mg, respectively, per 100 g body weight). Mice were then vaginally inoculated with 3x10⁵ IFU of *C. muridarum* in 20 µl of 2-SP buffer (200 mM sucrose, 12 mM K₂HPO₄, 8 mM KH₂PO₄) by pipette on three consecutive days to increase the likelihood that mice were in the diestrus stage of the reproductive cycle (62). The dose of *C. muridarum* was calculated based on the titer of the frozen stock determined as described above. Mice infected with *N. gonorrhoeae* alone and control mice were similarly anesthetized and mock-inoculated with 10 µl of 2-SP buffer. On the final day of *C. muridarum* inoculation, vaginal smears from all mice were prepared on glass slides and stained with a Hema-3 stain (Fisher Scientific) to identify mice in the diestrus stage as described (15). Mice with a predominance of PMNs and nucleated epithelial cells, rather than squamous epithelial cells, were considered to be in diestrus. Mice
found to be in diestrus were then treated with a subcutaneous injection of 0.5 mg of water-soluble 17β-estradiol (estradiol<sub>ws</sub>, Sigma) (76) approximately six hrs following the final inoculation with <em>C. muridarum</em> or buffer, and 2 and 4 days later. Four hrs after the second dose of estradiol<sub>ws</sub>, mice were vaginally inoculated with 1x10<sup>6</sup> colony forming units (CFU) of <em>N. gonorrhoeae</em> in 20 µl of phosphate buffered saline (PBS) (<em>N. gonorrhoeae</em> only and coinfected groups), or mock-inoculated with PBS (<em>C. muridarum</em> only and uninfected control groups). The <em>N. gonorrhoeae</em> inoculum was prepared as described elsewhere (32) and the dose confirmed by quantitative culture. All mice were given vancomycin hydrochloride (0.6 mg, twice daily) and streptomycin sulfate (2.4 mg, twice daily) via i.p. injection beginning two days before <em>N. gonorrhoeae</em> inoculation and maintained for seven days to control overgrowth of commensal flora. In two experiments, <em>C. muridarum</em> infection was allowed to occur for 8-10 days prior to treatment with estradiol<sub>ws</sub> and inoculation with <em>N. gonorrhoeae</em>. In these experiments, n = 5 and 10 and the combined results are reported.

**Quantitation of colonization load and PMN influx.** The number of gonococci and chlamydia recovered from each group was determined daily for 10 days in three separate experiments consisting of four groups (n = 10-11 mice per group in each experiment). Vaginal mucus was collected with a PBS-soaked polyester swab and a small portion of the sample was inoculated onto an HIA plate for recovery of facultatively anaerobic commensal flora and a glass slide for enumeration of PMNs. The percentage of PMNs per 100 vaginal cells was determined by cytological differentiation of stained vaginal cells as described (15). The remaining sample was then suspended in 1 ml of transport buffer (2-SP buffer supplemented with 3% FBS and 0.5 mg/ml vancomycin). Suspensions were cultured onto GC-VCNTS agar to isolate <em>N. gonorrhoeae</em> using the Autoplater 4000 (Spiral Biotech) and then frozen at -80°C for culture of
C. muridarum. The number of CFU of N. gonorrhoeae recovered was enumerated using the Spiral Biotech Q-Counter Software. For C. muridarum, suspensions were diluted and cultured onto monolayers of L929 cells and quantified by immunofluorescence as described above. The limits of detection were 20 CFU (N. gonorrhoeae) and 12.5 IFU (C. muridarum) per ml vaginal swab suspension. Upper genital tracts (uterine horns, oviducts, and ovaries) were cultured for N. gonorrhoeae and C. muridarum 10 days after inoculation with N. gonorrhoeae or PBS (chlamydia alone group) in a single experiment consisting of 12 mice per group. Upper genital tracts were homogenized in 2-SP buffer. The homogenate was serially diluted in PBS and cultured for N. gonorrhoeae on GC-VCNTS. The remaining homogenate was frozen at -80°C and C. muridarum titers were determined using a plaque assay (57) except that monolayers of L929 cells were used.

Enzyme-linked immunosorbent assay (ELISA) for C. muridarum- and N. gonorrhoeae-specific antibodies. Vaginal washes and sera were collected on days 10 and 28 post-challenge with N. gonorrhoeae (N. gonorrhoeae–infected and coinfectected groups) or PBS (uninfected and C. muridarum-infected groups) in the 3 experiments performed to assess gonococcal and chlamydial colonization and examined for chlamydia- or gonococcal-specific antibodies. EBs from C. muridarum strain Nigg were purified by Renograffin (Bracco Diagnostics) gradient (8) and outer membrane vesicles (OMVs) from N. gonorrhoeae strain FA1090 were prepared as described (7). Ninety-six well plates were coated with 50 µl of either C. muridarum EBs (5 µg/ml) or N. gonorrhoeae OMVs (10 µg/ml) in 0.5 M NaHCO₃ overnight at room temperature. All washes were performed with PBS containing 0.1% Tween-20 using the Molecular Devices Skan Washer. Wells were blocked with PBS containing 15% FBS for 30 min at 37°C in a humidified chamber. Sera were diluted 1:100 and 1:900 and vaginal washes were diluted 1:30.
and 1:100 in the block solution and added to the wells (50 µl) followed by incubation with secondary antibody (goat anti-mouse IgG, IgM, or IgA conjugated to horse radish peroxidase, Sigma) diluted 1:10,000 in the block solution. Incubations with primary and secondary antibodies were for one hr at 37°C in a humidified chamber. TMB-peroxidase Detection Solution (BioRad) was added to detect bound secondary antibody. The reaction was stopped after ten min with 0.1 N H₂SO₄ and the optical density at 450 nm was read using Biotek Instruments EL80 Universal Microplate Reader and KC Junior software.

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

Immunohistochemical tissue analysis. Tissue was collected for histological examination during a single experiment consisting of coinfected mice, mice infected with either pathogen alone, and uninfected control mice. Whole genital tracts were harvested from 5 mice per group on day 2 post-inoculation with *N. gonorrhoeae* or PBS (chlamydia alone), and tissue was fixed in 10% buffered formalin for 24 hrs and then stored in 70% ethanol prior to embedding in paraffin and sectioning onto slides for immunohistochemical analysis. Sections were double
immunolabeled for *C. muridarum* using the Chlam-III anti-chlamydial antibody (Santa Cruz Biotechnology) at a 1:50 dilution and mouse anti-serum raised against *N. gonorrhoeae* strain FA1090 outer membrane vesicles at a 1:500 dilution by Histoserv, Inc. (Germantown, MD).

**Tissue culture adherence assay.** ME180 human cervical epithelial cells were cultured as described above. IEC4.1 mouse intestinal epithelial and BM1.11 mouse oviduct cells [gifts of Drs. Harlan Caldwell (Rocky Mountain Laboratories, Hamilton, MT) and Raymond Johnson (Indiana University School of Medicine, Indianapolis, IN), respectively] were cultured as described elsewhere (69). Cells were seeded into 24-well plates and cultured to obtain monolayers of 80-90% confluency, and inoculated with *C. trachomatis* (ME180 cells) or *C. muridarum* (IEC4.1 and BM11.1 cells) at an MOI of 1 or mock-inoculated with SPG buffer with centrifugation in culture media, as described above. After 20 hr incubation in normal culture medium, *N. gonorrhoeae* strain FA1090 was diluted in PBS to an OD$_{600}$ of 0.07, followed by dilution in RPMI (Quality Biological) supplemented with 10% FBS and 0.3 μM Fe(NO$_3$)$_3$, and monolayers were inoculated with 500 μl of the *N. gonorrhoeae* suspension (final MOI = 1).

After 2 hrs at 37°C in 7% CO$_2$, the number of cell-associated gonococci was determined by washing monolayers 4 times with PBS followed by host cell lysis with 0.5% saponin in PBS and quantitative culture. Invasion was measured by the gentamicin (Gm) protection assay. For intracellular bacteria, monolayers were washed 2 times with PBS after 2 hrs incubation, and 500 μl RPMI supplemented with 10% FBS, 0.3 μM Fe(NO$_3$)$_3$, and 50 μg/ml Gm was added for 1.5 hours. Monolayers were washed 5 times with PBS and cells were lysed in 0.5% saponin in PBS followed by quantitative culture. Results are expressed as the percent of cell-associated bacteria relative to the inocula (% adherence) or as the percent of Gm-protected bacteria relative to the
number of adherent bacteria under the same conditions (% invasion). Conditions were performed in triplicate and each experiment was repeated at least three times.

**Gene expression analysis by RT-PCR.** Vaginal material was collected with a PBS-soaked polyester swab and suspended directly in 500 µl RNA-Later (Ambion) from uninfected or *C. muridarum*-infected mice 15 min prior to inoculation with *N. gonorrhoeae* to determine expression levels of the cathelicidin-related antimicrobial peptide (CRAMP) gene *cnlp*, and the secretory leukocyte peptidase inhibitor (SLPI) gene. Samples were stored at -80°C until use.

Total RNA was extracted using the Qiagen mini-RNeasy isolation kit per the manufacturer’s instructions. RNA was converted to cDNA using the SABioscience RT² EZ First Strand cDNA kit and then used for real-time PCR. cDNA reaction mixtures (20 µl total volume) were diluted to a final volume of 100 µl in nuclease-free water and 5 µl of diluted cDNA template was subjected to PCR amplification using an ABI 7500 sequence detector [25 µl total reaction volume consisting of template, 12.5 µl SYBR green master mix (ABI), and 10 µl primer mix, which contained both forward and reverse primers at concentrations of 1 µM]. Reactions were performed according to the following parameters: 10 min at 95°C, then 40 cycles at 95°C for 15 s and at 60°C for 1 min. The cycle threshold (Cₜ) value was determined using the Sequence Detector v.1.7a software (ABI). Data were analyzed with Microsoft Excel using the comparative Cₜ method (ΔΔCₜ) for relative quantification of gene expression using β-actin as the normalizer, as described (58). Expression of the CRAMP and SLPI genes was measured by normalizing to the β-actin gene. The calculation used included the difference between the Cₜ values of the normalizer (β-actin) and the Cₜ values of the test genes (*cnlp* and SLPI) in individual samples, as follows: ΔCₜ(C. *muridarum* infected or uninfected) = Cₜ(β-actin) - Cₜ(test gene) - ΔCₜ values for all uninfected mice were calculated and the mean of these values was used as a baseline for calculating the
\( \Delta \Delta C_T \) value for each *C. muridarum*-infected mouse. The relative difference in gene expression between each *C. muridarum*-infected mouse and the uninfected baseline value on day 0 (prior to inoculation with *N. gonorrhoeae*) was defined as the difference of normalized gene expression levels as follows: 

\[ 2^{\Delta \Delta C_T} = \Delta C_T(\text{uninfected baseline}) - \Delta C_T(\text{infected}) \]

The experiment was performed twice with 4-5 mice per group. The sequences of the oligonucleotide primers are shown in Table I.

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

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

Coinfection of female mice with *N. gonorrhoeae* and *C. muridarum*. Female mice are most susceptible to infection with *N. gonorrhoeae* during the estrogen-dominant stage of the estrous cycle (15, 35, 41) and *C. muridarum* during the progesterone-dominant stage of the estrous cycle (6, 79). Progesterone treatment is not required for long term colonization with *C. muridarum* provided that mice are inoculated during the diestrus stage of the estrous cycle (62). In contrast, susceptibility to long term colonization with *N. gonorrhoeae* requires administration of estradiol to promote an estrus-like state. In pilot experiments, our attempts to coinfect estradiol-treated female BALB/c mice with *N. gonorrhoeae* and *C. muridarum* simultaneously were unsuccessful. However, we found that once *C. muridarum* infection was established, subsequent treatment with estradiol did not cause chlamydial infection to clear or alter the number of chlamydial inclusions recovered, in agreement with previous observations (38). Thus, we developed a model in which chlamydial infection was established followed by inoculation with *N. gonorrhoeae* (Figure 1). Using this protocol viable gonococci and chlamydia were recovered from vaginal swabs for an average of 6-7 days and 9-10 days, respectively, post inoculation with *N. gonorrhoeae* in three separate experiments (Tables II and III). *N. gonorrhoeae* diplococci and *C. muridarum* inclusions were visualized in genital tract tissues of coinfected mice by immunohistochemical staining (Figure 2). *C. muridarum* inclusions were restricted to the cervix of coinfected animals. Gonococci were observed in the lumens of the vagina and the cervix as well as deep within the vaginal tissue at two days post-gonococcal challenge. We conclude that coinfection of female mice with *N. gonorrhoeae* and *C. muridarum* can be established.

We saw no difference in the rate of ascendant infection with either pathogen. Ascendant chlamydial infection was observed in 75% of *C. muridarum*-infected mice and this percentage
was not altered by coinfection with *N. gonorrhoeae* (mean of $10^3$ PFU of *C. muridarum* per upper genital tract in both groups; data not shown). This rate of ascension is comparable to rates seen in progesterone-treated C57/BL6 mice (56). No gonococci were recovered from the upper genital tract at day 10 post-gonococcal challenge in mice with or without a pre-existing chlamydial infection in these experiments.

**Estradiol treatment alters the host response to *C. muridarum***. The initial inflammatory response to *C. muridarum* infection in progesterone-treated mice is intense and wanes over time (16). We defined the kinetics of the inflammatory response to *C. muridarum* in our model system by investigating the effects of estradiol on the inflammatory response to *C. muridarum* alone in the absence of inoculation with *N. gonorrhoeae*. Mice were infected with *C. muridarum* or mock-inoculated with buffer (uninfected control) on three consecutive days. On the final day of *C. muridarum* inoculation, 10 diestrus-stage mice were identified and half of these mice were treated with estradiol, as per the usual protocol, and half were not (n = 5 mice per group). All diestrus-stage mice in the uninfected control group were treated with estradiol (n = 4 mice). Two days later, all mice were inoculated with PBS, rather than *N. gonorrhoeae*. Vaginal levels of seven different cytokines and chemokines (MIP-2, IL-1β, TNF-α, IFN-γ, RANTES, IL-10, and IL-17) that were previously implicated in gonococcal (11, 22, 25, 47, 58) or chlamydial infection (5, 16, 17, 63, 66, 83) were measured on the day of PBS challenge and over the next 10 days. Consistent with *C. muridarum* inducing an inflammatory response, high levels of IFN-γ, TNF-α, IL-1β, and MIP-2 were detected in mice that were not treated with estradiol on days 1-4 post-inoculation. MIP-2 and IL-1β levels remained elevated over the next 7 days, while IFN-γ and TNF-α levels declined at a time point that corresponds to day 4 post-inoculation with *N. gonorrhoeae* in our coinfection protocol (Figure 3A). Administration of estradiol dampened the
response to *C. muridarum* as evidenced by similar levels of IL-1β, TNFα, and IFNγ in estradiol-treated, infected (Figure 3B) and estradiol-treated, uninfected (Figure 3C) mice. Interestingly, estradiol treatment did not fully abrogate the MIP-2 response to *C. muridarum* as vaginal MIP-2 levels were elevated in estradiol-treated, infected mice compared to uninfected, estradiol-treated mice on days 2-8 post-PBS challenge (Figure 3C). The high levels of MIP-2 in estradiol-treated, uninfected mice on days 0 and 1 and at late time points (days 9 and 10) when the effects of estradiol begin to wear off reflect the normal fluctuations in MIP-2 that are associated with the influx of vaginal PMNs during the metestrus and diestrus phases of the estrous cycle (77). We conclude that the requirement to administer estradiol to establish gonococcal infection reduces the inflammatory response to chlamydial infection, but that significant levels of MIP-2 are still induced.

**Vaginal PMN influx is increased in coinfected mice.** An influx of PMNs is characteristic of symptomatic infection with *N. gonorrhoeae* or *C. trachomatis* in both women and men. Similarly, a localized PMN influx is observed in gonococcal infection of BALB/c mice (58, 76) and mouse models of chlamydial infection (17, 32, 60). To determine whether the PMN response is altered during coinfection, the percentages of PMNs in vaginal smears from estradiol-treated uninfected mice, coinfected mice, and mice infected with either *N. gonorrhoeae* or *C. muridarum* alone were compared. A similar percentage of PMNs was observed on stained vaginal smears from estradiol-treated mice infected with either *N. gonorrhoeae* or *C. muridarum* alone. In both groups, vaginal PMNs were detected beginning at day 5 post-challenge with *N. gonorrhoeae* (gonorrhea alone) or PBS (chlamydia alone group), and gradually increased with significantly higher percentages of PMNs detected in both groups on days 9 and 10 compared to uninfected mice (Fig. 4A, solid symbols). Vaginal PMNs were also observed in coinfected mice
beginning on day 5 post-inoculation with *N. gonorrhoeae*, with significantly higher percentages of PMNs observed in coinfected mice versus uninfected mice on days 5-10. On days 6, 7, and 8 post-inoculation with *N. gonorrhoeae*, a significantly higher percentage of vaginal PMNs were detected in coinfected mice compared to mice infected with either pathogen alone (Figure 4A, open squares versus solid symbols).

MIP-2, the mouse analog of the human PMN attracting chemokine IL-8, correlates with PMN influx at the infection site in both gonococcal and chlamydial mouse infection models (17, 58). Here, levels of MIP-2 in genital tract secretions also mirrored the degree of vaginal PMN influx, with MIP-2 levels beginning to increase in coinfected mice and mice infected with either pathogen alone on day 5 and continuing to increase during the period of significantly increased PMN influx for all infected groups (days 6-8). MIP-2 levels, like the percentage of vaginal PMNs, were highest in the coinfected group on days 6, 7, and 8 post-inoculation with *N. gonorrhoeae* (Figure 4B). MIP-2 levels were elevated in *C. muridarum*-infected (similar to that shown in Figure 3B) and in coinfected mice, but not *N. gonorrhoeae*-infected mice on days 0 and 1, but decreased to below the limit of detection by day 2. After day 8, MIP-2 levels began to increase in all groups, including uninfected mice, as the effects of estradiol treatment wore off and mice began cycling again (data not shown). Other cytokines and chemokines tested (IL-1β, TNFα, IFNγ, RANTES, IL-10, and IL-17) were not significantly increased in coinfected mice at time points corresponding to changes in PMN influx (data not shown). In summary, these results suggest that despite the demonstrated immunosuppressive effect of the estradiol used in this model, an inflammatory response that results in a localized PMN influx is generated against mice infected with either single pathogen, and that this response is intensified in mice that are coinfected with *C. muridarum* and *N. gonorrhoeae*.  

17
No difference was detected in the pathogen-specific antibody response. *C. muridarum-*infected mice respond robustly to infection and the antibody response peaks at approximately 4 weeks post-infection (3). In contrast, a weak antibody response occurs during *N. gonorrhoeae* murine infection, which is localized and transient and peaks at approximately 5 days post-infection (76). It is likely that increased or different interactions occur between innate receptors and bacterial ligands during coinfection compared to infection with either pathogen alone. We therefore hypothesized that coinfection may alter the humoral response to one or both pathogens.

As expected, we detected significant levels of serum *C. muridarum*-specific IgG and IgM and vaginal IgA and IgG in chlamydia-infected mice on days 10 and 28. However, we found no difference between chlamydia-infected and coinfect ed mice (Figure 5 and data not shown). We were unable to detect gonococcal-specific antibodies in vaginal washes from coinfect ed mice or mice infected with *N. gonorrhoeae* alone on day 10 or 28 post-gonococcal challenge; earlier time points may have shown detectable antibodies (76). We conclude that while coinfection alters the innate immune response, there is no evidence that it affects the adaptive response at the level of antibody production.

**Gonococcal colonization is increased in coinfect ed mice.** As discussed, we found no difference in the duration of infection with either pathogen in coinfect ed versus singly infected mice (Tables II and III). We also found no difference in the number of IFU of *C. muridarum* recovered from the lower genital tract of mice infected with *C. muridarum* alone or with both *C. muridarum* and *N. gonorrhoeae* (Figure 6A). However, a 0.5 - 1 log increase in the number of gonococci recovered from mice coinfect ed with *N. gonorrhoeae* and *C. muridarum* was observed as early as one day post-inoculation with *N. gonorrhoeae* compared to mice infected with *N. gonorrhoeae* alone (Figure 6B). This difference was maintained through approximately eight
days post-inoculation with *N. gonorrhoeae* and was highly reproducible. To investigate whether an increased gonococcal colonization load would be seen in mice that were infected with *C. muridarum* for a longer time period before challenging with *N. gonorrhoeae*, we postponed estradiol treatment to 6 days after the final inoculation of *C. muridarum*. Mice were thus infected with *C. muridarum* for 8-10 days prior to challenge with *N. gonorrhoeae*, as described in Materials and Methods, which is when the initial intense inflammatory response to *C. muridarum* wanes and cytokine levels were the most similar between estradiol-treated and untreated *C. muridarum*-infected mice (Fig. 3). In two separate experiments we again observed increased gonococcal colonization in mice with a pre-existing *C. muridarum* infection (Figure 6C). We conclude that the effect of *C. muridarum* infection on gonococcal colonization is sustained during infection and can occur after the period in which the inflammatory response to *C. muridarum* peaks.

*C. muridarum* does not affect *N. gonorrhoeae* association with cultured murine epithelial cells. To investigate the mechanism behind the increased gonococcal colonization load in *C. muridarum* coinfected mice, we used an *in vitro* coinfection model to determine whether gonococci are more able to associate with and invade chlamydia-infected epithelial cells. We hypothesized that chlamydial infection may alter the epithelial cell surface to allow greater gonococcal adherence or invasion, thus accounting for the increased gonococcal colonization load observed *in vivo*. Murine epithelial cell lines of intestinal and oviduct origin were infected with *C. muridarum* or left uninfected and incubated for 20 hrs prior to inoculation with *N. gonorrhoeae*, which is the time point that coincides with active RB replication and the peak epithelial inflammatory cytokine response to chlamydial infection observed *in vitro* (65). Human cervical epithelial cells infected with *C. trachomatis* were tested similarly. We observed
no difference in the percent of cell-associated or invasive gonococci in either human or murine
epithelial cells in the presence or absence of *C. trachomatis* or *C. muridarum* infection,
respectively (Figure 7). Interestingly, the level of gonococcal adherence to human and murine
cell lines was similar despite the host restrictions associated with the human-specific pathogen *N.
gonorrhoeae* (Figure 7A). However, the level of gonococcal invasion into human cells was
much greater than in either of the murine cell lines (Figure 7B), underscoring the importance of
the host-restricted receptors in mediating gonococcal invasion of host cells.

*C. muridarum* infection alters the immune environment. Because we observed a difference in
gonococcal colonization as early as day one post-inoculation with *N. gonorrhoeae*, we
hypothesized that immunological differences may exist as a result of chlamydial infection that
could confer a more hospitable environment for *N. gonorrhoeae*. We therefore measured the
concentration of seven different cytokines and chemokines in vaginal swab suspensions that are
reported to play a role in both gonococcal and chlamydial infection (MIP-2, IL-1β, TNF-α, IFN-
γ, RANTES, IL-10, and IL-17) in *C. muridarum* infected or mock-infected (control) mice at the
time point that corresponds to inoculation with *N. gonorrhoeae*. Significant increases in the
localized concentrations of the inflammatory mediators MIP-2 and TNF-α were detected in
chlamydia-infected versus control mice (Figure 8).

Several reports suggest that levels of MIP-2 and TNF-α can be kept in check by
antimicrobial peptides, such as cathelicidin-related antimicrobial peptide (CRAMP) and
secretory leukocyte peptidase inhibitor (SLPI), which also function as immunomodulatory
molecules (30, 44, 51). Both CRAMP and SLPI are known to be effective against Gram-
negative organisms and thus, their absence may help to explain an increase in gonococcal
colonization (42). Therefore, we measured the expression of these two genes in chlamydia-
infected and uninfected mice by RT-PCR. Transcripts for CRAMP and SLPI were approximately two-fold reduced in *C. muridarum*-infected mice at the time point that corresponds to inoculation with *N. gonorrhoeae* (Figure 9). These data support the hypothesis that chlamydia-induced alterations in the immune environment of the lower genital tract make the genital tract more permissive for *N. gonorrhoeae.*
Discussion

The incidence of chlamydia and gonorrhea coinfection in the young adult population in the United States is very high as illustrated by a survey of 18-26 year olds in which approximately 70% of young adults with gonorrhea were also infected with *Chlamydia trachomatis* (50). Despite these startling numbers, little is currently known about the pathogenesis or host response to coinfection. Advances are needed in this area to reduce both the costs associated with presumptive dual antibiotic treatment and the consequences of coinfection on reproductive health. Here, we describe the first small animal model of gonococcal and chlamydial coinfection for studying host-parasite interactions specific to coinfection and for developing products that are effective against both agents. While characterizing this model, we found that coinfection of female mice differs from infection with either pathogen alone in terms of gonococcal colonization load and host response to infection.

Successful coinfection of mice with *N. gonorrhoeae* and *C. muridarum* required that we first establish chlamydial infection in the absence of exogenous hormone treatment, as described (62), and then treat with 17β-estradiol to promote susceptibility to *N. gonorrhoeae*. This sequence of infection is likely to mimic a common scenario that leads to coinfection of women as suggested by mathematical modeling of two cohort studies, which showed a large number of women are asymptomatically infected with *C. trachomatis* for 18 months or more in the absence of antibiotic treatment (24). The average length of gonococcal infection is thought to be much shorter (28). Thus, it is likely that most women with coinfection at diagnosis were either asymptomatically colonized with *C. trachomatis* for a long period of time prior to infection with *N. gonorrhoeae* or acquired both organisms simultaneously from a coinfected partner. An obvious limitation to this model is the need to treat mice with estradiol to promote susceptibility.
to infection with *N. gonorrhoeae*, which we showed reduces the inflammatory response to *C. muridarum*. Female mice are differentially susceptible to many different sexually transmitted pathogens depending on stage of the estrous cycle, and therefore, mouse models of sexually transmitted infections frequently utilize hormone treatment including models of *N. gonorrhoeae* (32), *C. muridarum* (3, 6), *C. trachomatis* (79), *Mycoplasma genitalium* (49), *Candida albicans* (71), and herpes simplex virus-2 (61) infections. Here we showed that several cytokines and chemokines and vaginal PMNs were decreased in *C. muridarum*-infected mice following treatment with estradiol. This model therefore does not mimic the events that occur when women with symptomatic chlamydial infection encounter *N. gonorrhoeae*. Chlamydial infection of estradiol-treated mice may more closely mimic asymptomatic chlamydial infection in women, however, which is likely often the case when a *C. trachomatis*-infected woman encounters *N. gonorrhoeae* (24). In support of this possibility, Agrawal and colleagues observed lower levels of several cytokines and chemokines in cervical washes from *C. trachomatis*-infected asymptomatic women compared to women who were symptomatic for infection (2).

Additional limitations to the model we describe here include the use of *C. muridarum* instead of *C. trachomatis* and host restrictions inherent to the use of the human-specific pathogen *N. gonorrhoeae* in a murine system. The host response and progression of infection and disease in mice infected with *C. muridarum* more closely mimics human disease, however, than does experimental infection of mice with *C. trachomatis* (3, 16), and the *C. muridarum* mouse model is commonly used to examine the immunobiology of chlamydial genital infection. Host restrictions that prevent murine infection with *N. gonorrhoeae* from fully mimicking human infection include the absence of colonization receptors for pili and opacity (Opa) proteins (13, 36, 52, 80) and differences in soluble complement regulatory proteins that bind the gonococcal...
surface to downregulate complement activation (54). *N. gonorrhoeae* also cannot use murine lactoferrin or transferrin as sources of iron (14, 43), and the gonococcal immunoglobulin A1 (IgA1) protease cannot cleave mouse IgA (40). Despite these host restrictions, studying gonococcal pathogenesis in the murine model has yielded considerable insight into the host response to infection (25, 31, 58, 76) and the role of certain gonococcal virulence factors in evasion of host defenses (34, 75, 81, 84, 85). The mouse model has also allowed the demonstration of hormonal influences on selection of phase variable Opa proteins *in vivo* (13, 32, 73) as well as the effect of certain antibiotic resistance mutations on microbial fitness (82). The increasing availability of transgenic mice in several of these host-restricted factors should allow for improved study of gonococcal chlamydial coinfection *in vivo*.

An important finding of our current study was the demonstration that higher numbers of *N. gonorrhoeae* colonized the murine genital tract when *C. muridarum* was present. This result was observed whether mice were infected with *C. muridarum* for a short period (2-4 days) or a longer interval (8-10 days) prior to challenge with *N. gonorrhoeae*. We interpret this finding as evidence that the factors responsible for increased gonococcal colonization are sustained during chlamydial infection. There are several possible explanations for the observed increased gonococcal colonization in *C. muridarum*-infected mice, including potential differences in the availability of nutrients, colonization receptors, or innate defenses. Experiments with tissue culture cells did not support the hypothesis that *C. muridarum* alters the number of gonococci that adhere to or invade epithelial cells. We also saw no difference in the number of gonococci required to infect mice with a pre-existing chlamydial infection as might be predicted if *C. muridarum* infection enhanced the capacity of gonococci to adhere to epithelial cells in the initial stages of infection (R.A. Vonck and A.E. Jerse, unpublished observation). We therefore
examined the hypothesis that host responses to *C. muridarum* may alter the immune response to *
*N. gonorrhoeae*. In support of this hypothesis we observed increased levels of the inflammatory
mediators MIP-2 and TNF-α in chlamydia-infected mice prior to inoculation with *N.
antimicrobial peptides CRAMP and SLPI. Decreased levels of antimicrobial peptides, one of the
first lines of defense at the mucosal surface, could contribute to the increased gonococcal
colonization that we observed during coinfection. The report that vaginal fluids from women
with *C. trachomatis* had reduced levels of SLPI (21), and the demonstration that *N. gonorrhoeae*
is susceptible to CRAMP and the human cathelicidin LL37 *in vitro* are consistent with this
hypothesis (72, 82). This hypothesis is supported further by a growing body of evidence that IL-
17 responses (25) and TLR4-mediated responses (59), both of which lead to the production of
antimicrobial peptides (26, 86), are protective against *N. gonorrhoeae*. Optimization of methods
to detect and measure antimicrobial peptide concentrations in genital tract secretions, which is
currently underway in our laboratory, should facilitate more in-depth studies on the
consequences of altered antimicrobial peptide responses during coinfection.

MIP-2 levels correlate with localized PMN influx in both *N. gonorrhoeae* (58) and *C.
muridarum* (17) infection models and a second important difference between coinfection and
infection with either pathogen alone was the higher vaginal levels of MIP-2 and greater PMNs
influx in coinfected mice. Interestingly, in a recent study of patients who were admitted to
genitourinary medicine clinics in the United Kingdom, women that were coinfected with *C.
trichomatis* and *N. gonorrhoeae* were more likely to be symptomatic than women infected with
*C. trachomatis* alone (68). The mechanisms responsible for induction of greater levels of
inflammation in coinfected mice and humans remain to be elucidated. The simplest explanations
are that higher concentrations of pro-inflammatory pathogen-derived ligands, due to the presence of both chlamydiae and gonococci, may have a cumulative effect or that the higher gonococcal colonization load in coinfected mice may influence the degree of PMN influx. It is also possible that a unique interplay occurs between distinct pathogen-specific signaling pathways, which results in greater inflammation. At this time only the PMN response has been investigated, and the involvement of other inflammatory cell types and signaling pathways during coinfection warrant further investigation. Other potential consequences of coinfection that could be examined in this model include the possibility that *N. gonorrhoeae* infection may reactivate a latent chlamydia infection as suggested by Batteiger et al. (4) in a study of recurrent chlamydial infections. The coinfection mouse model should also facilitate the development of immunomodulatory therapies, such as toll-like receptor (TLR) agonists and antagonists, which have been proposed to be used along with antimicrobial treatment to prevent the devastating effects of sexually transmitted infections on women’s reproductive health (29).

Perhaps the largest potential consequence of the unique characteristics of coinfection that we describe here is on transmission. Studies performed by Cohen and colleagues demonstrated a dose response for experimental urethral infection of male volunteers in which the percentage of infected volunteers increased with increasing dose of *N. gonorrhoeae* (12). Thus, increased gonococcal colonization due to chlamydial infection may lead to increased transmission from a coinfectected female to a male partner. Additionally, the spread of *Chlamydia* both to the upper reproductive tract of the infected host and to an uninfected partner may be facilitated by the host PMN response, as proposed by Rank and colleagues from studies with *C. caviae* in a guinea pig genital tract infection model (64). The increased PMN influx observed in coinfectected female mice might then be expected to lead to increased transmission from a coinfectected female to a male partner.
partner. Three published studies have attempted to determine whether there is a difference in the rate \textit{N. gonorrhoeae} and \textit{C. trachomatis} transmission to an uninfected partner in the context of either single or dual infection, and no clear difference in transmission of either organism in the case of coinfection was identified (45, 46, 48). However, investigators in these studies were unable to appropriately identify index cases and several patients included as index cases were actually negative for an infection for which their partner tested positive. Thus, as concluded by Matondo and colleagues in 1995, we believe this question requires further study (48).

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

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Table I. *Oligonucleotide sequences used in this study*

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<th>Gene</th>
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<td>GCAGATCTACTGTCGGCTGAGGTA</td>
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<td>SLPI</td>
<td>CGGCTCTGGACTCGTGCTCGG</td>
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<td>(74)</td>
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<td>β-actin</td>
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<td>CATCGTACTCCTGTTGCTG</td>
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Table II. Coinfection does not alter the duration of recovery of *N. gonorrhoeae*

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<th>Mean Duration of Bacterial Recovery (Range)$^a$</th>
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<tr>
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<td><em>N. gonorrhoeae</em> Alone</td>
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<tr>
<td>Experiment I</td>
<td>6.4 (1-10)</td>
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<tr>
<td>Experiment II</td>
<td>6.1 (2-9)</td>
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<tr>
<td>Experiment III</td>
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<tr>
<td><strong>Cumulative</strong></td>
<td><strong>6.6 (1-10)</strong></td>
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</tbody>
</table>

$^a$Values represent the mean number of days out of 10 consecutive days *N. gonorrhoeae* was recovered by vaginal swab from mice in three separate experiments with the range in days represented in parenthesis.
Table III. *Coinfection does not alter the duration of recovery of C. muridarum*

<table>
<thead>
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<th></th>
<th>Mean Duration of Bacterial Recovery (Range)(^a)</th>
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<td></td>
<td><em>C. muridarum Alone</em></td>
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<tr>
<td>Experiment I</td>
<td>9.2 (8-10)</td>
</tr>
<tr>
<td>Experiment II</td>
<td>9.9 (9-10)</td>
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<td>Experiment III</td>
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<tr>
<td><strong>Cumulative</strong></td>
<td><strong>9.5 (5-10)</strong></td>
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</tbody>
</table>

\(^a\)Values represent the mean number of days out of 10 consecutive days *C. muridarum* was recovered by vaginal swab from mice in three separate experiments with the range in days represented in parenthesis.
**Figure 1.** Time line for coinfection protocol. Mice were inoculated with *C. muridarum* on three consecutive days to establish chlamydial infection. Following the final inoculation with *C. muridarum*, mice in the diestrus stage of the estrous cycle were treated with water-soluble estradiol and inoculated with a single dose of *N. gonorrhoeae* two days later. In the experiments described, mice were infected with *C. muridarum* for either 2-4 days or 8-10 days prior to inoculation with *N. gonorrhoeae*. 
**Figure 2.** Immunohistochemical staining reveals the presence of *N. gonorrhoeae* and *C. muridarum* in the coinfected genital tract. (A) Genital tract tissue extracted from a coinfected mouse on day 2 post-*N. gonorrhoeae* inoculation at 40X magnification. Boxes labeled B and C correspond to the regions shown in panels B and C, respectively, where they are magnified at 400X. (B) Cervical tissue at 400X magnification showing distinct *C. muridarum* inclusions (gray arrows) within nucleated epithelial cells. Panels C, D, E, and F show tissue from the same mouse at 400X magnification with visible *N. gonorrhoeae* diplococci (black arrows) among squamous epithelial cells in the vaginal lumen (C), squamous epithelial cells in the vaginal lumen and superficially associated with the vaginal epithelium (D), deep within the vaginal tissue (E), and in the lumen of the cervix (F). Several chlamydial inclusions and a single diplococcus are shown in the insets of higher magnification in panels B and D, respectively. All scale bars represent 50 µm. The dual staining performed by Histoserv, Inc. was done with a different color chromagen for each organism for positive identification.
**Figure 3.** The host inflammatory response to *C. muridarum* infection is altered by estradiol treatment. Protein concentrations (pg/ml) of cytokines and chemokines in genital tract secretions from (A) non-estradiol treated, *C. muridarum* infected mice, (B) estradiol-treated, *C. muridarum* infected mice, and (C) estradiol-treated, uninfected mice (n = 4-5 mice per group).
Figure 4. A greater PMN influx occurs in coinfected mice. (A) Significantly more PMNs migrate into the lower genital tract of mice coinfected with *N. gonorrhoeae* and *C. muridarum* (white squares, solid line) compared to mice infected with *N. gonorrhoeae* (black circles, dashed line) or *C. muridarum* (black squares, dashed line) alone, or uninfected mice (white circles, solid line) on days 6, 7, and 8 post-*N. gonorrhoeae* inoculation (*p < 0.05*). Percent PMNs is defined as (number of PMNs counted)/(100 vaginal cells observed, including squamous and nucleated epithelial cells and leukocytes). Results shown are from three combined experiments (*n* = 30 – 32 mice per group). (B) Levels of PMN attracting chemokine MIP-2 are greatest on days 6-8 in mice coinfected with *N. gonorrhoeae* and *C. muridarum* (white squares, solid line) compared to mice infected with either *N. gonorrhoeae* (black circles, dashed line) or *C. muridarum* (black squares, dashed line) alone or uninfected mice (white circles, solid line) on days 6-8 (*p < 0.05, coinfected vs. uninfected; # p < 0.05, coinfected vs. *C. muridarum* alone). Results from a single experiment are shown (*n* = 4 – 5 mice per group). The high average level of MIP-2 detected on day 5 in *C. muridarum*-infected mice is due to one mouse in this group having 1,843 pg/ml of MIP-2. MIP-2 concentrations ranged from 60-646 pg/ml for the other four mice within this group.
Figure 5. Coinfection does not alter the level of *C. muridarum*-specific antibodies. Levels of *C. muridarum*-specific serum IgG (A) and vaginal IgA (B) were measured by ELISA and the absorbance at 450 nm is shown for samples collected from mice infected with *C. muridarum* alone, mice coinfected with *C. muridarum* and *N. gonorrhoeae*, mice infected with *N. gonorrhoeae* alone, and uninfected mice in a single representative experiment. Samples were collected on day 28 post-inoculation with *N. gonorrhoeae*. Sera samples were diluted 1:900 and vaginal washes were diluted 1:100. Symbols indicate values for individual mice; horizontal bars show the geometric mean.
Figure 6. *C. muridarum* infection alters levels of *N. gonorrhoeae* in the lower genital tract. (A) *C. muridarum* colonization remained constant whether mice were coinfected with *N. gonorrhoeae* (white squares, solid line) or infected with *C. muridarum* alone (black squares, dashed line) and (B) *N. gonorrhoeae* colonization was greater in mice coinfected with *C. muridarum* (white squares, solid line) than in mice infected with *N. gonorrhoeae* alone (black circle, dashed line) (*p* = 0.011 by repeated measures ANOVA). Results shown in panels A and B are from 3 combined experiments (n = 31-32 mice per group). (C) A similar trend in *N. gonorrhoeae* colonization during coinfection with *C. muridarum* was observed when mice with a longer pre-established *C. muridarum* infection were challenged with *N. gonorrhoeae* (n = 11-19 mice per group). The time point at which mice were challenged with *N. gonorrhoeae* in the experiment shown in panel C corresponds to day 6 in panels A and B. Results are expressed as log_{10} inclusion forming units (IFU) or colony forming units (CFU) per ml of vaginal swab suspension.
**Figure 7.** Chlamydial coinfection does not result in increased association of gonococci with cultured epithelial cells. Shown is the percentage of cell-associated (A) or invasive (B) gonococci following inoculation of monolayers of ME180, IEC4.1, or BM1.11 cells with *N. gonorrhoeae* in the presence (white bars) or absence (gray bars) of a pre-existing chlamydial infection. ME180 human cervical epithelial cells were pre-infected with *C. trachomatis* and the IEC4.1 and BM1.11 murine epithelial cells were pre-infected with *C. muridarum.*
Figure 8. Vaginal levels of inflammatory mediators MIP-2 and TNFα are increased in *C. muridarum*-infected mice on day 0, prior to inoculation with *N. gonorrhoeae*. Concentrations (pg/ml) in genital tract secretions of mice infected with *C. muridarum* (solid bars) and uninfected mice (white bars) are shown from a single experiment (n = 9-10 mice per group, *p < 0.05*).
Figure 9. Antimicrobial peptide gene expression is down-regulated in mice with a pre-existing chlamydial infection prior to inoculation with *N. gonorrhoeae*. CRAMP and SLPI gene expression levels were measured by RT-PCR on vaginal material collected from mice with and without a pre-existing chlamydial infection 15 minutes prior to inoculation with *N. gonorrhoeae*. Each dot represents an individual *C. muridrum*-infected mouse as compared to the average baseline value in uninfected mice at the same time point. The line is drawn at the geometric mean with the numerical value in parenthesis. The dashed line represents a fold change of one, which is the value that corresponds to no difference between mice with and without a pre-existing chlamydial infection. Values less than one indicate down-regulation of gene expression in *C. muridrum*-infected mice.
C. muridarum inoculation (or buffer control)

N. gonorrhoeae inoculation (or buffer control)
<table>
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