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RESEARCH ARTICLE

Neisseria gonorrhoeae Coinfection during *Chlamydia muridarum* Genital Latency Does Not Modulate Murine Vaginal Bacterial Shedding

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ABSTRACT *Chlamydia trachomatis* and *Neisseria gonorrhoeae* are the most frequently reported agents of bacterial sexually transmitted disease worldwide. Nonetheless, *C. trachomatis*/*N. gonorrhoeae* coinfection remains understudied. *C. trachomatis*/*N. gonorrhoeae* coinfections are more common than expected by chance, suggesting *C. trachomatis*/*N. gonorrhoeae* interaction, and *N. gonorrhoeae* infection may reactivate genital chlamydial shedding in women with latent (quiescent) chlamydial infection. We hypothesized that *N. gonorrhoeae* would reactivate latent genital *Chlamydia muridarum* infection in mice. Two groups of *C. muridarum*-infected mice were allowed to transition into genital latency. One group was then vaginally inoculated with *N. gonorrhoeae*; a third group received *N. gonorrhoeae* alone. *C. muridarum* and *N. gonorrhoeae* vaginal shedding was measured over time in the coinfected and singly infected groups. Viable *C. muridarum* was absent from vaginal swabs but detected in rectal swabs, confirming *C. muridarum* genital latency and consistent with the intestinal tract as a *C. muridarum* reservoir. *C. muridarum* inclusions were observed in large intestinal, but not genital, tissues during latency. Oviduct dilation was associated with *C. muridarum* infection, as expected. Contradicting our hypothesis, *N. gonorrhoeae* coinfection did not reactivate latent *C. muridarum* vaginal shedding. In addition, latent *C. muridarum* infection did not modulate recovery of vaginal viable *N. gonorrhoeae*. Evidence for *N. gonorrhoeae*-dependent increased *C. muridarum* infectivity has thus not been demonstrated in murine coinfection, and the ability of *C. muridarum* coinfection to potentiate *N. gonorrhoeae* infectivity may depend on actively replicating vaginal *C. muridarum*. The proportion of mice with increased vaginal neutrophils (PMNs) was higher in *N. gonorrhoeae*-infected than in *C. muridarum*-infected mice, as expected, while that of *C. muridarum*/*N. gonorrhoeae*-coinfected mice was intermediate to the singly infected groups, suggesting latent *C. muridarum* murine infection may limit PMN response to subsequent *N. gonorrhoeae* infection.

IMPORTANCE Our work builds upon the limited understanding of *C. muridarum*/*N. gonorrhoeae* coinfection. Previously, *N. gonorrhoeae* infection of mice with acute (actively replicating) vaginal *C. muridarum* infection was shown to increase recovery of viable vaginal *N. gonorrhoeae* and vaginal PMNs, with no effect on *C. muridarum* vaginal shedding (R. A. Vonck et al., Infect Immun 79:1566–1577, 2011). It has also been shown that chlamydial infection of human and murine PMNs prevents normal PMN responses, including the response to *N. gonorrhoeae* (K. Rajeeve et al., Nat Microbiol 3:824–835, 2018). Our findings show no effect of latent genital

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C. muridarum infection on the recovery of viable *N. gonorrhoeae*, in contrast to the previously reported effect of acute *C. muridarum* infection, and suggesting that acute versus latent *C. muridarum* infection may have distinct effects on PMN function in mice. Together, these studies to date provide evidence that *Chlamydia*/*N. gonorrhoeae* synergistic interactions may depend on the presence of replicating *Chlamydia* in the genital tract, while chlamydial effects on vaginal PMNs may extend beyond acute infection.

KEYWORDS *Chlamydia*, *Neisseria gonorrhoeae*, coinfection, mouse model

Chlamydia trachomatis and *Neisseria gonorrhoeae* are the agents of most common bacterial sexually transmitted infections (STIs) worldwide, with 129 million new cases of chlamydia and 82 million new cases of gonorrhea reported in 2020 (1). These obligate human pathogens cause cervicitis in women and urethritis in men, with frequent asymptomatic and extragenital infection. Both can ascend to the upper genital tract, leading to pelvic inflammatory disease (PID), chronic abdominal pain, infertility, and ectopic pregnancy (2). *C. trachomatis* treatment failure is common (3, 4), progressive *N. gonorrhoeae* antimicrobial resistance is widespread (5), and no vaccines are available, despite decades of effort (6). The development of new *C. trachomatis* and *N. gonorrhoeae* therapies and vaccines is of critical public health concern.

Obligate intracellular *C. trachomatis* alternates between infectious elementary bodies (EBs) and replicative reticulate bodies (RBs). Inside host cells, EBs reside in membrane-bound inclusions and differentiate into RBs. After replication, RBs differentiate again into EBs and are released (7). *Chlamydia* induces innate and adaptive immune responses that may progress for months to years. The inflammatory response, important for infection resolution, also contributes to tissue damage and disease (8). Early in chlamydial infection, rapid neutrophil (PMN) influx is not sufficient to clear, but may limit, infection (9). *C. trachomatis* can survive inside PMNs, limiting PMN function (10). *N. gonorrhoeae* adheres to mucosal epithelia, forming microcolonies, and is capable of invasion and transcytosis, which can lead to disseminated infection (11). *N. gonorrhoeae* also recruits PMNs to the site of infection and can survive within PMNs. *N. gonorrhoeae* modulation of PMN function may also serve to facilitate *N. gonorrhoeae* growth and transmission (11–13).

C. trachomatis/*N. gonorrhoeae* coinfection may increase transmissibility and long-term complications. High rates of *C. trachomatis* coinfection occurred among young women with *N. gonorrhoeae* (14), and *N. gonorrhoeae* shedding was higher in *C. trachomatis*-coinfected women than in those with only *N. gonorrhoeae* (15). *N. gonorrhoeae* coinfection was a risk factor for recurring female *C. trachomatis* infection (16), which is associated with PID and reproductive complications (17). Notably, *N. gonorrhoeae* infection may reactivate latent (e.g., inapparent, undetected) *C. trachomatis* infection. Female contacts of men with *C. trachomatis*-negative, *N. gonorrhoeae*-positive urethritis had twice the chlamydial infection incidence of female contacts of men with *C. trachomatis*-negative, *N. gonorrhoeae*-negative urethritis (18). Recurring *C. trachomatis* infection with the same chlamydial serovar was also associated with *N. gonorrhoeae* coinfection (19). Similarly, 22% of female sex partners of *N. gonorrhoeae*-only infected men were *C. trachomatis* infected, further supporting that coinfection with *N. gonorrhoeae* may reactivate latent female *C. trachomatis* infection (20).

C. trachomatis/*N. gonorrhoeae* pathogenesis studies have focused largely on single pathogen infections (21–24). *Chlamydia muridarum* murine genital tract infection, used to model infection in women, induces inflammatory response and genital pathology similar to human disease (25). Experimental *N. gonorrhoeae* infection of estradiol-treated BALB/c mice is characterized by cervicovaginal infection with inflammatory PMN infiltration (26). The sole *C. muridarum*/*N. gonorrhoeae* coinfection mouse model showed higher viable *N. gonorrhoeae* vaginal load and vaginal PMNs in *C. muridarum*/*N. gonorrhoeae*-coinfected mice compared to *N. gonorrhoeae*-only infected mice (27). It was later shown that *C. trachomatis* infection of *ex vivo*

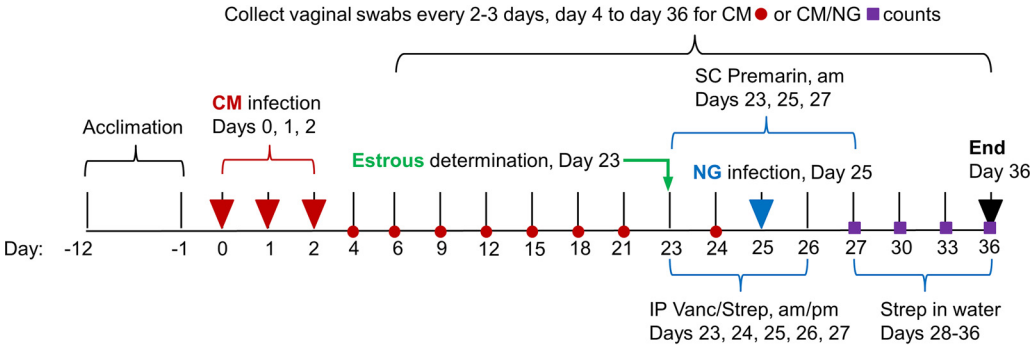

FIG 1 Experimental design. Mice were vaginally inoculated with *C. muridarum* (labeled "CM" in the figure) daily for three consecutive days. Viable *C. muridarum* shedding and recovery of viable *N. gonorrhoeae* (labeled "NG" in the figure) were monitored by regular vaginal swabbing, every 2 to 3 days, for the duration of the experiment. During chlamydial latency, vaginal smears were collected to determine estrous cycle stage, and selected mice (anestrus and diestrus stage) were treated with subcutaneous (labeled "SC" in the figure) injections of Premarin and intraperitoneal (labeled "IP" in the figure) injections of antibiotics (vancomycin hydrochloride and streptomycin sulfate [Vanc/Strep]) for 4 days, as shown. Two days after initiation of Premarin/antibiotics, mice were vaginally inoculated once with *N. gonorrhoeae*. After the cessation of antibiotic injections, Strep was supplied in drinking water. Mice that were not selected for Premarin/antibiotics treatment were sacrificed at day 23 (early sacrifice), and all other mice were sacrificed at day 36 (late sacrifice). At sacrifice, rectal swabs were collected for viable *C. muridarum* enumeration, and necropsy was performed to evaluate gross genital tract pathology; genital and intestinal tracts tissues were collected for evaluation of pathology.

human PMNs reduced subsequent *N. gonorrhoeae*-induced NET formation while increasing *N. gonorrhoeae* survival in PMNs (10). More recently, in a coinfection tissue culture model, we showed that *N. gonorrhoeae* elicited antichlamydial effects that were consistent with epithelial host cell sphingolipid depletion, suggesting *C. trachomatis*/*N. gonorrhoeae* interaction via host cell factors (28). These three studies, to our knowledge, are the only published experimental *Chlamydia*/*N. gonorrhoeae* coinfection studies. Although providing evidence of *Chlamydia*/*N. gonorrhoeae* interactions, they did not evaluate latent chlamydial infection.

We aimed here to evaluate the effect of *N. gonorrhoeae* coinfection specifically on latent *C. muridarum* infection. We hypothesized *N. gonorrhoeae* coinfection would reactivate latent genital chlamydial infection. We show (i) that *N. gonorrhoeae* does not reactivate *C. muridarum* from genital latency; (ii) that latent genital chlamydial infection, unlike acute genital chlamydial infection (27), does not increase the recovery of viable vaginal *N. gonorrhoeae*; and (iii) that *C. muridarum* infection may limit subsequent *N. gonorrhoeae*-dependent luminal vaginal PMN increase, consistent with chlamydial inhibition of PMN function (10). Our findings, together with previous studies, suggest differing effects of acute versus latent chlamydial infection on subsequent murine *N. gonorrhoeae* infection. Continued evaluation of *Chlamydia*/*N. gonorrhoeae* interaction, and how such coinfection may impact the host, is important to inform improved interventions for chlamydia and gonorrhea.

RESULTS

Study design and model characteristics. Previously, acute *C. muridarum* genital infection increased recovery of viable *N. gonorrhoeae* and the influx of vaginal PMNs when *N. gonorrhoeae* infection was initiated 2 to 8 days after *C. muridarum* infection (27). Given that inapparent female *C. trachomatis* infection may be reactivated by *N. gonorrhoeae* (18–20), we hypothesized that murine *N. gonorrhoeae* coinfection during chlamydial genital latency would reactivate latent *C. muridarum* infection. We delayed *N. gonorrhoeae* infection until 23 days after the final *C. muridarum* inoculation (Fig. 1) to coincide with chlamydial genital latency, as characterized by undetectable *C. muridarum* vaginal shedding, but continued intestinal infection. Mice in estrus have limited susceptibility to *C. muridarum* infection via vaginal inoculation (29), and inoculation for three consecutive days ensures mice are exposed to *C. muridarum* during the luteal stage of the 4- to 5-day cycle. Inoculation with 10^5 to 10^7 inclusion forming units (IFU)

of *C. muridarum* typically results in peak infection around 10^4 to 10^5 IFU/vaginal swab, lasting 15 to 21 days (30). In a pilot study (n = 10 mice), 3 days of vaginal inoculation with 10^5 IFU/day resulted in limited infection (50% of mice), with 10^1 to 10^4 maximum IFU/swab detectable for only ~1 week (data not shown). Therefore, we used an inoculum dose of 10^6 *C. muridarum* IFU.

We monitored vaginal *C. muridarum* shedding to confirm decline to genital latency. To confirm mice harbored viable *C. muridarum* and infection had not been cleared, we monitored rectal shedding of *C. muridarum*, since the intestinal tract may serve as a *C. muridarum* reservoir for recrudescent vaginal infection (31, 32). Mice were then evaluated for estrous stage, and those in anestrus or diestrus were treated with Premarin and antibiotics to facilitate robust, extended *N. gonorrhoeae* infection (23, 27). After vaginal *N. gonorrhoeae* coinfection, we monitored vaginal viable *C. muridarum* shedding and recovery of viable *N. gonorrhoeae* to determine impact of coinfection, comparing singly infected (*C. muridarum* or *N. gonorrhoeae*) versus *C. muridarum*/*N. gonorrhoeae*-coinfected groups. We collected genital and intestinal tract tissues for pathology analyses and immunohistochemical (IHC) analyses for *C. muridarum* and *N. gonorrhoeae* in these tissues, and we used vaginal smears to enumerate vaginal luminal PMNs.

The study consisted of three independent experiments (10 to 40 mice each, 90 total mice; see File S1 in the supplemental material). Mice were divided into four groups: coinfected with *C. muridarum* and *N. gonorrhoeae*, infected with either pathogen alone (*C. muridarum* or *N. gonorrhoeae*) or control, mock-infected (UN). One mouse died spontaneously on day 1, prior to completion of *C. muridarum* infection, with no pathological findings, and was excluded from analyses. No other mice showed negative impact on well-being (see File S2). On day 23, mice not in anestrus or diestrus, and thus suboptimal for Premarin treatment (33), were sacrificed ("early sacrifice"). Mice in anestrus or diestrus remained in the study until the end of experiment and were Premarin/antibiotic treated, mock or *N. gonorrhoeae* inoculated, and sacrificed on day 36 ("late sacrifice"). Mice without detectable *C. muridarum* or *N. gonorrhoeae* shedding were excluded from vaginal bacterial shedding (but not pathology) analyses (see File S1 for the numbers of mice in each group).

In the first independent experiment (experiment 1, n = 10 mice; see File S1 and Table S1 in the supplemental material), we compared *C. muridarum* viable vaginal shedding (*C. muridarum* titer) and *C. muridarum* genome (*Chlamydia* qPCR, *C. muridarum* qPCR) positivity for all vaginal swabs collected (n = 106 swabs). Two mice were from the early sacrifice on day 23, coinciding with *C. muridarum* genital latency prior to subsequent *N. gonorrhoeae* infection (one mock infected and one *C. muridarum* infected); the eight remaining mice were sacrificed on day 36, at the end of experiment (four *N. gonorrhoeae* and four *C. muridarum*+*N. gonorrhoeae*). As expected, all swabs from mice not infected with *C. muridarum* were titer and qPCR negative. Swabs collected from *C. muridarum*-infected mice on days 4 to 21 (n = 31 swabs) were 45.2% *C. muridarum* titer and qPCR positive. Eleven swabs were determined to be positive by titer and qPCR, three swabs were positive by titer only, three swabs were positive by qPCR only. All swabs from days 24 to 36 were titer and qPCR negative. Given approximately equivalent *C. muridarum* titer and qPCR positivity, vaginal swab *C. muridarum* genomes were not monitored for the remainder of the study.

Vaginal *N. gonorrhoeae* coinfection does not reactivate latent genital chlamydial shedding. The primary outcome evaluated was the number of viable bacteria recovered from vaginal swabs. We hypothesized latent genital *C. muridarum* shedding would be reactivated by *N. gonorrhoeae* coinfection. Shedding was assessed by quantitative titer assay, and analyzed and presented as mean "*C. muridarum* shedding" in $\log_{10}$ *C. muridarum* IFU/swab (standard deviation [SD]) and mean "*N. gonorrhoeae* recovery" in $\log_{10}$ *N. gonorrhoeae* CFU/swab (SD) (Fig. 2). Until day 24 of the study, mice in the *C. muridarum* and *C. muridarum*+*N. gonorrhoeae* groups exhibited similar expected acute vaginal shedding. Shedding peaked on day 4 and became undetectable by day 24, indicating genital latency (Fig. 2A). The day 4 mean $\log_{10}$ (SD) IFU/swab for *C. muridarum* was 3.66 (2.29), and that for *C. muridarum*+*N. gonorrhoeae* was 3.49

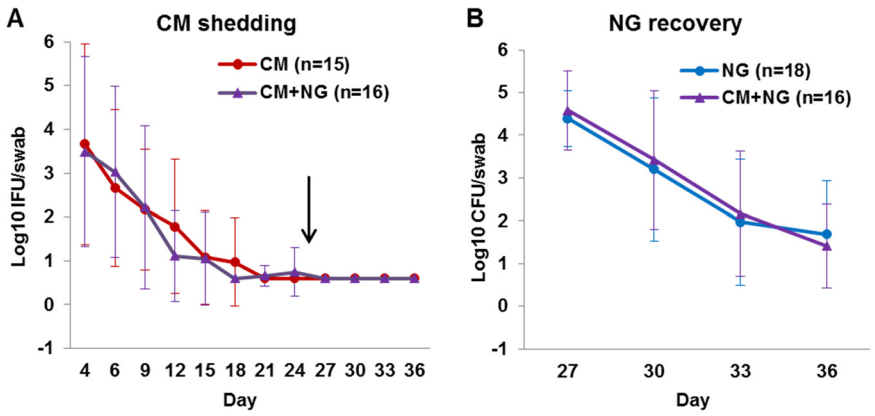

FIG 2 Vaginal viable *C. muridarum* (labeled "CM" in the figure) and *N. gonorrhoeae* (labeled "NG" in the figure). (A and B) Quantification of viable *C. muridarum* shedding was determined by infecting LLC cells (A), and quantification of viable *N. gonorrhoeae* recovery was determined by culturing on commercial agar plates selective for *N. gonorrhoeae* (B). The results are expressed as mean $\log_{10}$ IFU or CFU per vaginal swab ± the SD. The arrow in panel A indicates *N. gonorrhoeae* vaginal inoculation on day 25.

(2.17); the result for both groups combined was 3.57 (2.17). The day 4 $\log_{10}$ IFU/swab ranges were as follows: *C. muridarum* = 0.6 to 7.06, *C. muridarum*+*N. gonorrhoeae* = 0.6 to 6.55, and all *C. muridarum* infected = 0.6 to 7.06.

Detection of *C. muridarum* vaginal shedding began primarily on day 4 (83.9%) and in fewer mice on day 6 (9.7%) or day 9 (6.5%) (Table 1). Mice had detectable *C. muridarum* shedding in one to six vaginal swabs of the twelve swabs collected. All mice ceased detectable shedding by day 24, including those excluded due to the lack of detectable *N. gonorrhoeae* vaginal shedding. No mice in the UN or *N. gonorrhoeae* groups had detectable viable *C. muridarum* vaginal shedding in any swabs (see Materials and Methods for the contamination check protocol). Thus, although *C. muridarum* vaginal shedding declined to genital latency as expected, in contradiction to our hypothesis, *N. gonorrhoeae* coinfection failed to cause resumed *C. muridarum* shedding in latently *C. muridarum*-infected mice (Fig. 2A). Critically, preliminary experiments showed that it is possible for *C. muridarum* vaginal shedding to be reactivated from an undetectable level upon immune suppression in our mouse model (BALB/c mice and *C. muridarum* Weiss strain; see File S3).

Latent *C. muridarum* genital infection is associated with rectal viable chlamydial shedding. We evaluated viable *C. muridarum* rectal shedding in swabs collected during necropsy at early and late sacrifice. In addition, *C. muridarum* qPCR analysis of rectal swabs was performed for all *C. muridarum*-inoculated mice (Table 2; see also File S1). As expected, all early-sacrifice mock-infected mice and all late-sacrifice UN and *N. gonorrhoeae*-infected mice showed no detectable viable *C. muridarum* rectal shedding (see Materials and Methods for contamination check protocol).

All *C. muridarum*-infected early-sacrifice mice were both *C. muridarum* rectal titer and rectal qPCR positive 2 days prior to *N. gonorrhoeae* infection (day 25), indicating that the mice had not cleared *C. muridarum* infection (Table 2). Late-sacrifice mice were analyzed whether or not they had detectable *C. muridarum* or *N. gonorrhoeae* vaginal shedding, and rectal *C. muridarum* positivity ranged from approximately 20 to

TABLE 1 Viable *C. muridarum* vaginal shedding

	No. of positive mice/total no. of mice (%)								
Infection	Day 4	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 24	Days 27–36
C. muridarum	11/15 (73.3)	12/15 (80)	12/15 (80)	7/15 (46.7)	4/15 (26.7)	2/15 (13.3)	0/15	0/15	0/15
C. muridarum+*N. gonorrhoeae*	15/16 (93.8)	12/16 (75)	11/16 (68.8)	5/16 (31.3)	3/16 (18.8)	0/16	1/16 (6.3)	1/16 (6.3)	0/16
C. muridarum infected	26/31 (83.9)	24/31 (77.4)	23/31 (74.2)	12/31 (38.7)	7/31 (22.6)	2/31 (6.5)	1/31 (3.2)	1/31 (3.2)	0/31

TABLE 2 Mice with *C. muridarum* rectal carriage

Infection	*C. muridarum* titer		*C. muridarum* qPCR	
	No. of positive mice/total no. of mice (%)	Mean $\log_{10}$ (SD) *C. muridarum* IFU/rectal swab	No. of positive mice/total no. of mice (%)	Mean $\log_{10}$ (SD) *C. muridarum* genomes/rectal swab
Early sacrifice				
Mock infected	0/12		0/12	
C. muridarum infected	13/13 (100)	2.89 (0.62)	13/13 (100)	3.50 (0.75)
Late sacrifice				
Mock infected	0/4		0/4	
C. muridarum	4/15 (26.7)	0.84 (0.45)	9/15 (60)	1.79 (1.10)
N. gonorrhoeae	0/24		0/24	
C. muridarum +*N. gonorrhoeae*	5/21 (23.8)	0.88 (0.64)	8/21 (38.1)	1.34 (1.02)

60%; no mice with undetectable *C. muridarum* ($n = 1$) or *N. gonorrhoeae* ($n = 4$) vaginal shedding were *C. muridarum* rectal titer or qPCR positive. There was no difference in mean *C. muridarum* IFU/rectal swab or in mean *C. muridarum* genomes/rectal swab between *C. muridarum* and *C. muridarum*+*N. gonorrhoeae* groups, nor were the proportions of titer- or qPCR-positive mice significantly different for the *C. muridarum* versus the *C. muridarum*+*N. gonorrhoeae* groups. Thus, we found no indication that *N. gonorrhoeae* coinfection modulates latent rectal *C. muridarum* shedding.

Latent chlamydial genital infection does not modulate the recovery of viable *N. gonorrhoeae*. On days 27 to 36, *N. gonorrhoeae*-infected and *C. muridarum*+*N. gonorrhoeae*-infected mice exhibited robust and prolonged vaginal *N. gonorrhoeae* colonization similar to that previously described (27). Viable *N. gonorrhoeae* recovery peaked on day 27, followed by gradual decline until day 36. Specifically, the mean $\log_{10}$ (SD) CFU/swab on day 27 for *N. gonorrhoeae* was 4.39 (0.65), and that for *C. muridarum*+*N. gonorrhoeae* was 4.58 (0.92); the mean $\log_{10}$ (SD) CFU/swab on day 36 for *N. gonorrhoeae* was 1.68 (1.25), and that for *C. muridarum*+*N. gonorrhoeae* was 1.41 (0.98). Individual day 27 and day 36 $\log_{10}$ (SD) CFU/swab ranges were *N. gonorrhoeae* = 3.32 to 5.67 and *C. muridarum*+*N. gonorrhoeae* = 3.08 to 5.71 and were *N. gonorrhoeae* = 2.39 to 4.12 and *C. muridarum*+*N. gonorrhoeae* = 1.29 to 4.06, respectively (Fig. 2B).

N. gonorrhoeae and *C. muridarum*+*N. gonorrhoeae* groups had detectable *N. gonorrhoeae* shedding in one to four of the four vaginal swabs collected after *N. gonorrhoeae* infection; at least 25% of mice in both groups had detectable shedding on day 36 (Table 3). Viable *N. gonorrhoeae* vaginal shedding in the *N. gonorrhoeae* and *C. muridarum*+*N. gonorrhoeae* groups was detected starting on day 27. No mice in the UN or *C. muridarum* groups showed detectable viable *N. gonorrhoeae* shedding in any swabs (see Materials and Methods for the contamination check protocol). Mean viable *N. gonorrhoeae* vaginal shedding did not differ significantly between the *N. gonorrhoeae* and *C. muridarum*+*N. gonorrhoeae* groups (Fig. 2B). Thus, in contrast to the previous report that acute genital *C. muridarum* infection enhances vaginal shedding of live *N. gonorrhoeae* during subsequent *N. gonorrhoeae* coinfection (27), we found no evidence that latent genital *C. muridarum* infection can exert a similar effect on *N. gonorrhoeae* infection in this mouse model.

Genital tract *N. gonorrhoeae*, but not *C. muridarum*, was detected during chlamydial genital latency. For all fixed tissue analyses, early-sacrifice mice (i.e., sacrificed *before N. gonorrhoeae* inoculation) were evaluated as two groups: mock infected and *C. muridarum* infected. Late-sacrifice mice, i.e., sacrificed *after N. gonorrhoeae* or mock inoculation on day 25, were analyzed as UN, *C. muridarum*, *N. gonorrhoeae*, and *C. muridarum*+*N. gonorrhoeae* groups. By IHC, *C. muridarum* inclusions were not observed in the genital tracts of any mice, including those determined to be positive by *Chlamydia* (*C. muridarum*) qPCR evaluation of genital tissue (see Table S2). Genital tract tissue determined to be negative by

TABLE 3 Mice with viable *N. gonorrhoeae* vaginal shedding

Infection	No. of positive mice/total no. of mice (%)			
	Day 27	Day 30	Day 33	Day 36
N. gonorrhoeae	18/18 (100)	13/18 (72.2)	7/18 (38.9)	5/18 (27.8)
C. muridarum+*N. gonorrhoeae*	16/16 (100)	12/16 (75)	7/16 (43.8)	4/16 (25)

C. muridarum qPCR was not evaluated by *C. muridarum* IHC. In addition, of ten *C. muridarum* mice from a pilot study with 10^5 IFU inocula (data not shown), two, both early sacrifice, were genital tract *C. muridarum* qPCR positive but *C. muridarum* IHC negative.

Neisseria (*N. gonorrhoeae*) IHC was performed on 77 mice, including all *N. gonorrhoeae*-inoculated (e.g., *N. gonorrhoeae* and *C. muridarum*+*N. gonorrhoeae* late-sacrifice) mice for which vaginal tissue was available for analysis ($n = 42$; see Table S3). As expected, the genital tracts of mice not *N. gonorrhoeae* inoculated were *N. gonorrhoeae* IHC negative. Approximately 20 to 40% of *N. gonorrhoeae*-inoculated late-sacrifice mice were *N. gonorrhoeae* IHC positive, and the proportion of positive mice did not differ between the two groups. Positive *N. gonorrhoeae* labeling was present in the vagina and not other genital tract anatomical sites, as expected, since human transferrin is required for ascended *N. gonorrhoeae* infection in mice (34). *N. gonorrhoeae* vaginal positivity was primarily luminal, attached to the surface epithelium or to detached/luminal cornified epithelial cells (Fig. 3).

***Chlamydia* infection of the large intestine during latent genital chlamydial infection.** Given the observed rectal swab *C. muridarum* positivity, duodenum, jejunum, ileum, cecum, colon, and rectum tissues of *C. muridarum*-infected early-sacrifice and *C. muridarum* and *C. muridarum*+*N. gonorrhoeae* late-sacrifice mice with *C. muridarum* qPCR-positive intestinal tissue ($n = 16$; see Table S4) were investigated by *C. muridarum* IHC. If a single *C. muridarum* inclusion was observed, the mouse was considered *C. muridarum* IHC positive. Of the *C. muridarum* qPCR-positive mice, >50% ($n = 9$), all early-sacrifice *C. muridarum*-infected mice, were IHC positive (Table 4). Inclusions per intestinal cross section ranged from 1 up to 10 to 15 (data not shown). *C. muridarum* inclusions were observed in the intestinal epithelium of the cecum, colon, and/or rectum (large intestine), but not in the duodenum, jejunum, or ileum (small intestine) (Table 4). Examples of positive *C. muridarum* IHC are shown in Fig. 4.

Gross pathology and histopathology of the genital tract. Gross genital tract pathology of all mice was unremarkable (genital tracts were similar in appearance across all groups, including uninfected controls, and showed no marked swelling, redness, or other abnormalities). For all mice, one to three block sections per fixed genital tissue block were prepared to help facilitate assessment of the full genital tract (longitudinal section). The ovary, missing from sections of five mice, had a similar appearance across all experimental groups and was not further studied. The uterus, available but similar in appearance in all mice, showing only estrus cycle-dependent lumen dilation (mild, moderate, or severe), was not further evaluated (data not shown).

At least one oviduct cross section was present in at least one genital block section for all except two mice, which were excluded from analysis. Oviduct cross sections/mouse ranged from 1 to 17 (mean = 6.4, SD = 4.0), and dilation was typically observed in 1 to 2 cross sections/mouse (Fig. 5). The proportion of mice with dilation ranged from approximately 16 to 40%, and the proportion of mice with inflammatory infiltrates ranged from approximately 4 to 26% across all groups. Inflammatory infiltrates were localized periductal, mostly mild, and focal and consisted of a mixture of inflammatory cells such as PMNs, macrophages, lymphocytes, or plasma cells (Fig. 5). Perioviductal inflammation was associated with oviduct dilation in six mice. Among the early-sacrifice mice, 20% with oviduct dilation also had periductal inflammation, while 33.3% with periductal inflammation also had oviduct dilation. Among the late-sacrifice mice, 31.25% with oviduct dilation also had periductal inflammation, while 62.5% with periductal inflammation also had oviduct dilation. *C. muridarum*-induced hydrosalpinx was not observed.

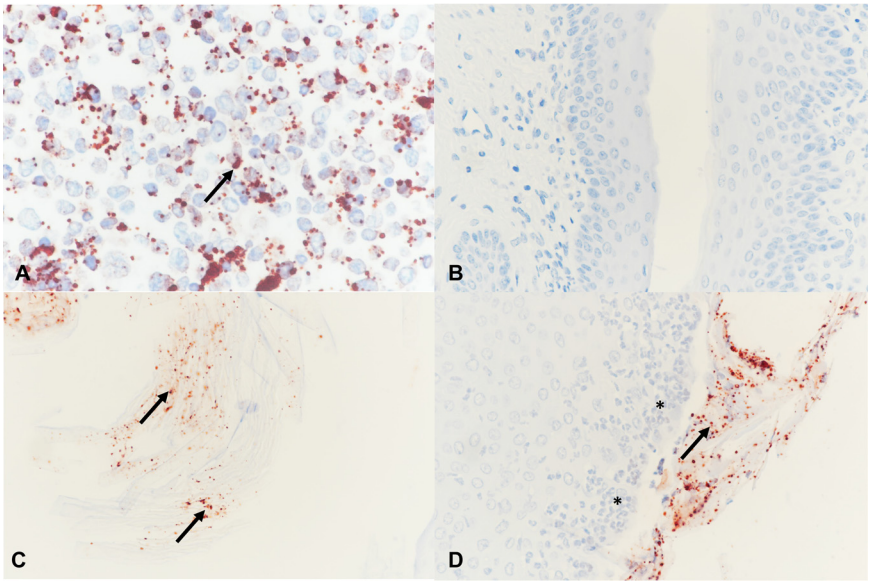

FIG 3 *Neisseria* (*N. gonorrhoeae*) immunohistochemistry (IHC) was performed in the positive-control cell pellet (A) and in mouse vagina (B to D) with red positively labeled extracellular *N. gonorrhoeae* (arrows). (A) Cell pellet infected with *N. gonorrhoeae*. (B) Vagina negative IHC, late-sacrifice *N. gonorrhoeae* group. (C) Luminal *N. gonorrhoeae* attached to cornified epithelial cells, late-sacrifice *C. muridarum*+*N. gonorrhoeae* group. (D) *N. gonorrhoeae* attached to superficial layers of the vaginal epithelium, late-sacrifice *N. gonorrhoeae* group. The vaginal epithelium is infiltrated with PMNs (asterisks). Magnification, ×400.

The cervix, present in 85 mice, was lined by columnar epithelium transitioning to stratified epithelium. Cervical PMN luminal accumulation and/or intraepithelial infiltration, was graded as none, mild, moderate, or severe. The proportion of mice with cervical PMN accumulation/infiltration ranged from approximately 27 to 41% across all groups (see Table S6 in the supplemental material). PMN intraepithelial infiltration (n = 17) was more common than combined luminal/intraepithelial accumulation/infiltration (n = 10) or luminal accumulation (n = 1). Accumulation/infiltration ranged from mild (n = 13) to moderate (n = 6) to severe (n = 9) and did not differ between luminal and intraepithelial sites when present at both (Fig. 6).

The vagina, present in 84 mice, was mostly lined by a keratinized squamous epithelium depending on estrus stage (primarily estrus-like). Vaginal PMN luminal accumulation and/or intraepithelial infiltration, was graded as none, mild, moderate, or severe. The proportion of mice with vaginal PMN accumulation/infiltration ranged from approximately 40 to 75% across all groups (see Table S7). PMN intraepithelial infiltration (n = 31) was more common than combined luminal/intraepithelial accumulation/infiltration (n = 16) or luminal accumulation (n = 1). Accumulation/infiltration ranged from mild (n = 21) to moderate (n = 16) to severe (n = 11) and did not differ between luminal and intraepithelial sites when present at both (Fig. 6).

In 20 mice, cervical and vaginal PMN infiltration was present simultaneously. In early-sacrifice mice, 100% (of 9) of mice with cervical inflammation had concomitant

TABLE 4 Positive *Chlamydia* intestinal tract immunolabeling per anatomical localization

Anatomical localization	Positive immunolabeling (no.)
Cecum only	2
Colon only	1
Rectum only	2
Cecum + colon	2
Cecum + rectum	1
Colon + rectum	1
Total	9

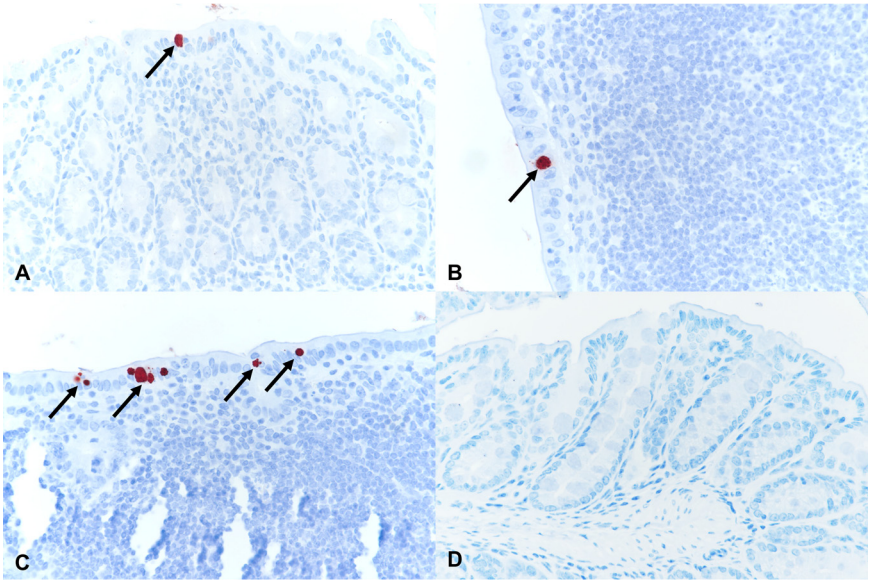

FIG 4 *Chlamydiaceae* immunohistochemistry in mouse intestines with red positively labeled intracytoplasmic chlamydial inclusions (arrows) in the intestinal epithelium of the early-sacrifice *C. muridarum*-infected group. (A) Cecum, early-sacrifice *C. muridarum*-infected group. (B) Colon, early-sacrifice *C. muridarum*-infected group. (C) Rectum, early-sacrifice *C. muridarum*-infected group. (D) Negative rectum, late-sacrifice *C. muridarum*+*N. gonorrhoeae* group. Magnification, ×400.

vaginal inflammation, while 9/15 (60%) with vaginal inflammation had cervical inflammation. In late-sacrifice mice, 11/19 mice (57.9%) with cervical inflammation had concomitant vaginal inflammation, while 11/32 (34.4%) had cervical inflammation.

Statistical analysis was performed on histopathology data for early-sacrifice mice, comparing mock-infected and *C. muridarum*-infected groups, and late-sacrifice mice, comparing UN, *C. muridarum*, *N. gonorrhoeae*, and *C. muridarum*+*N. gonorrhoeae* groups, minus any mice with missing relevant sections. Five pathology outcomes were considered: oviduct dilation ratio (dilated/total oviduct cross sections), oviduct dilation score (none, mild, or moderate), and oviduct inflammation (yes versus no), as well as cervical and vaginal infiltration with PMNs graded from 0 to 3 (none, mild, moderate, or severe). No outcomes were statistically different between groups (see Materials and Methods for details of statistical analyses).

Latent genital *C. muridarum* infection may limit subsequent *N. gonorrhoeae*-dependent increases in vaginal luminal PMNs. The proportion of mice with vaginal tissue inflammation was not increased in coinfected versus single-infected mice (see Table S7). Therefore, we evaluated vaginal luminal PMN numbers by microscopic analysis of vaginal smears (n = 64 late-sacrifice mice). Vaginal smears were categorized as having either no/rare PMNs (consistent with estradiol/estrogen-induced estrus; negative for increased vaginal luminal PMNs) or few/more PMNs (consistent with PMN influx into the vaginal lumen; positive for increased vaginal luminal PMNs). The relative proportions of mice with few/more PMNs were ~25% for the UN and *C. muridarum* groups, 65% for the *N. gonorrhoeae* group, and <50% for the *C. muridarum*+*N. gonorrhoeae* group. For the three infected groups, differences between proportion of positivity approached statistical significance, as determined by Fisher exact test (P = 0.05332). Pairwise comparisons, however, approached significant differences only for the *N. gonorrhoeae* and *C. muridarum* groups (P = 0.06743), while remaining pairwise comparisons yielded P values of >0.3. These observed trends (in vaginal tissues and vaginal smears) suggest that *C. muridarum*/*N. gonorrhoeae*-coinfected mice have a proportion of increased vaginal luminal PMNs intermediate to that of singly infected mice.

DISCUSSION

C. trachomatis/*N. gonorrhoeae* interaction may increase host susceptibility and/or transmissibility, the latter potentially due to a *C. trachomatis*-mediated increase in *N. gonorrhoeae*

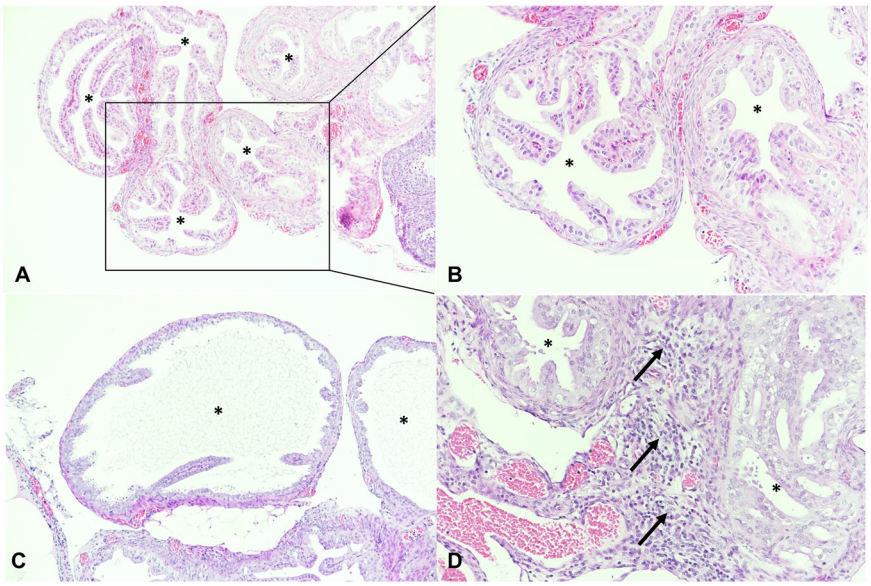

FIG 5 H&E staining of oviducts (asterisks). (A and B) Normal oviduct cross sections, late-sacrifice *N. gonorrhoeae* group. Magnifications, ×100 (A) and ×200 (B, see inset A). (C) Dilated oviducts (asterisk), late-sacrifice *C. muridarum* group. Magnification, ×100. (D) Perioviductal inflammation (arrows), mostly lymphocytes, early-sacrifice *C. muridarum*-infected group. Magnification, ×200.

bacterial load (15, 27). *N. gonorrhoeae* may increase incident *C. trachomatis* infection (35) and/or reactivate latent female genital chlamydial shedding (18–20), and *C. trachomatis*/*N. gonorrhoeae* coinfection may promote symptoms and negative health outcomes (35, 36). Three published coinfection studies exist to date, two providing *in vivo* and *ex vivo* evidence that active *Chlamydia* infection may influence *N. gonorrhoeae* infection (10, 27), while our more recent study showed *N. gonorrhoeae* antichlamydial effects in an epithelial *in vitro* model (28). Novel models and continued study of *C. trachomatis*/*N. gonorrhoeae* coinfection, in the context of productive and latent *Chlamydia* infection, will improve understanding of *C. trachomatis*/*N. gonorrhoeae* interaction.

Mice vaginally infected with *C. muridarum* (previously referred to as *C. trachomatis* MoPn, [37]) naturally progress to quiescent genital latency, and detectable vaginal *C. muridarum* shedding can be reactivated by pharmacological immune suppression (38; see also File S3 in the supplemental material). We hypothesized that *N. gonorrhoeae* coinfection would reactivate vaginal shedding in mice with latent genital *C. muridarum* infection, considering such reactivation may occur in women (18–20), and evaluated this hypothesis by modifying the sole existing mouse *C. muridarum*/*N. gonorrhoeae* coinfection model (27). Mice showed expected early peak viable vaginal *C. muridarum* shedding waning to undetectable levels, characteristic of genital latency (30, 38), and mice sacrificed at this time were 100% positive for viable rectal *C. muridarum* carriage. Thus, prior to *N. gonorrhoeae* infection, mice were not entirely cured of *C. muridarum* infection, having potential for reactivation of *C. muridarum* vaginal shedding, consistent with the intestine as a *C. muridarum* reservoir in mice (31, 32). Resumed vaginal *C. muridarum* shedding was not detected upon *N. gonorrhoeae* coinfection, however, contradicting our hypothesis.

Previous studies showed vaginal *C. muridarum* inoculation resulted in robust genital and intestinal infection (39), while intragastric or rectal *C. muridarum* inoculation resulted in intestinal infection, without autoinoculation of the genital tract, even after 70 days (40). Vaginal inoculation with *C. muridarum* attenuated for upper genital tract pathology, followed by intragastric wild-type *C. muridarum* coinoculation, also did not result in *C. muridarum* spread to the genital tract (41). It remains possible that *C. muridarum* intestinal infection could serve as a source for reactivation, though undetectable *C. muridarum* genital infection and/or genital persistent/aberrant chlamydial forms (42) may be the source of reactivated latent *C. muridarum* (38).

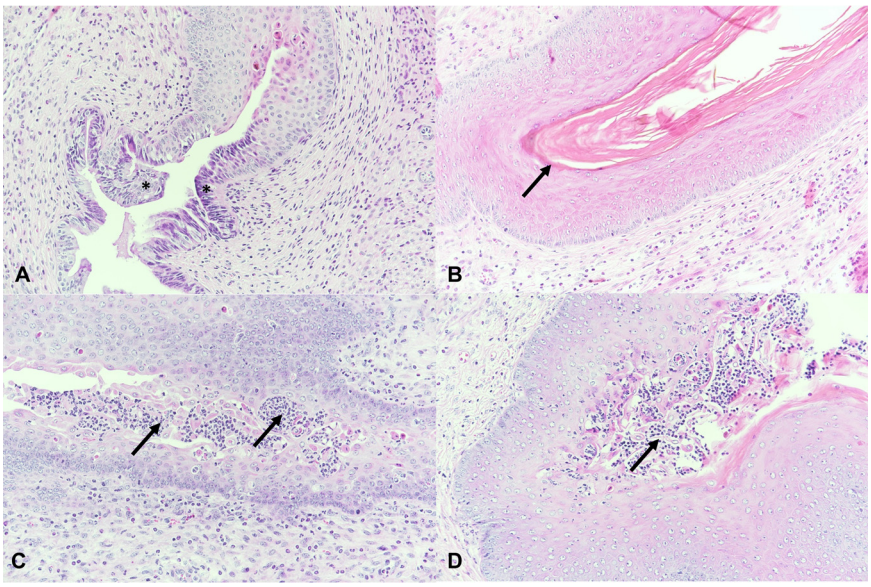

FIG 6 H&E staining of cervix and vagina. (A) Normal cervix with transition from columnar to stratified epithelium (asterisks); late-sacrifice *N. gonorrhoeae* group. (B) Normal vagina lined by keratinized squamous epithelium (arrow); late-sacrifice *N. gonorrhoeae* group. (C) Cervix, severe infiltration with PMNs (arrows), late-sacrifice *C. muridarum* group. (D) Vagina, severe infiltration with PMNs (arrows), late-sacrifice *N. gonorrhoeae* group. Magnification, ×200.

In our study, *C. muridarum* singly infected and *C. muridarum*/*N. gonorrhoeae*-coinfected groups showed similar *C. muridarum* infection characteristics prior to *N. gonorrhoeae* infection, largely precluding confounding differences in early *C. muridarum* infection. However, a major limitation of our study is the requirement for estradiol/estrogen treatment to promote *N. gonorrhoeae* infection, given that this induced state of estrus is likely unfavorable for *C. muridarum* genital infection. Similar estradiol treatment in the original *C. muridarum*/*N. gonorrhoeae* coinfection model did not prevent acute vaginal *C. muridarum* infection and viable shedding, nor was acute *C. muridarum* shedding increased by *N. gonorrhoeae* coinfection (27). However, we cannot rule out estrogen-limited *C. muridarum* susceptibility playing a role in the failure of *N. gonorrhoeae* coinfection to elicit reactivation of viable vaginal *C. muridarum* shedding in mice with latent genital *C. muridarum* infection.

In contrast to acute *C. muridarum* genital infection increasing subsequent recovery of viable vaginal *N. gonorrhoeae* (27), we did not observe a similar *N. gonorrhoeae*-enhancing effect for latent genital *C. muridarum* infection. This may indicate, since there is no detectable *C. muridarum* vaginal shedding in genital *C. muridarum* latency, that actively replicating *C. muridarum* must be present in the murine vagina to elicit the effect on vaginal *N. gonorrhoeae*. In our recent *in vitro* study of genital epithelial cell coinfection, *N. gonorrhoeae* limited chlamydial development/infectivity in a manner consistent with host sphingolipid depletion; this effect also required live *N. gonorrhoeae* (killed *N. gonorrhoeae* and lysates failed to have an effect), providing an example of *N. gonorrhoeae*/*C. trachomatis* interaction via host cell factors that requires concomitant viability of both bacteria (28). In addition, in *in vitro* coinfection, *N. gonorrhoeae* CFU/mL was not increased in *C. trachomatis*/*N. gonorrhoeae*-coinfected versus *N. gonorrhoeae* singly infected samples; however, only a single final CFU/mL measurement of nonadherent and noninvaded *N. gonorrhoeae* present in the culture medium was made at the experimental endpoint (28).

Previously, in acute murine *C. muridarum* genital infection, *C. muridarum* inclusions were found in the cervix and *N. gonorrhoeae* was found in the cervix and vagina 2 days after *N. gonorrhoeae* infection, when live vaginal shedding for both bacteria was ~3 $\log_{10}$ IFU or CFU/swab (27). In contrast, we observed *N. gonorrhoeae* only in the vagina, and no *C. muridarum* inclusions in the genital tract, at 11 days after *N. gonorrhoeae* infection and 34 days after *C. muridarum* infection, presumably due to increased time

postinfection. Notably, acute *C. muridarum* genital infection was previously associated with increased levels MIP-2 and TNF-α (inflammatory mediators) and decreased transcription of two antimicrobial peptides (CRAMP and SLPI) prior to *N. gonorrhoeae* infection, the latter of which may explain acute *C. muridarum*-dependent enhancement of *N. gonorrhoeae* vaginal shedding (27). While we did not evaluate antimicrobial peptide transcription, it is possible they are important for the effect of acute genital *C. muridarum* infection on *N. gonorrhoeae* vaginal shedding, but are no longer modulated during *C. muridarum* genital latency.

Genital tract pathology in our progesterone-free coinfection model was milder than generally reported for progesterone-enhanced *C. muridarum* murine vaginal infection, which typically induces hydrosalpinx (43, 44). We assessed pathology 34 days after *C. muridarum* infection, and genital pathology measures are often made later, even up 80 days postinfection (43). In progesterone-assisted *C. muridarum* infection, hydrosalpinx can be observed by 35 days postinfection (45). Previously, progesterone-free *C. muridarum* vaginal infection in BALB/c mice also failed to show hydrosalpinx (46), so mouse strain and hormone-free *C. muridarum* infection may play a role. A large *C. muridarum* inoculum (10^5 to 10^7 IFU, similar to our inoculum of 10^6) has been shown to result in *less* ascending infection and hydrosalpinx (47). Furthermore, long-term stress increased *C. muridarum* load and pathology (48, 49), so tunnel handling as in our study (50, 51) may have reduced stress compared to standard tail-handling experiments, though we did not perform physiological or behavioral measures to support this.

C. muridarum in vitro passage may reduce hydrosalpinx formation and severity (52). Our passage of *C. muridarum* in LLC cells, as opposed to commonly used HeLa, McCoy, or Hec1B cells (43, 47, 53), might thus have also influenced pathogenicity. Though we observed peak shedding and course of decline to latency similar to that previously reported for progesterone-assisted vaginal inoculation of BALB/c mice with Hec1B-propagated *C. muridarum* (53), we cannot rule out an effect of propagation parameters. Notably, we observed no genital tissue-associated pathology associated with coinfection. We observed background oviduct dilation, cervix inflammatory cells, and especially, vaginal inflammatory cells, which may be due to the frequent vaginal manipulations (inoculation, swabbing), intraperitoneal antibiotic administration or the effects of antibiotics on vaginal normal flora. However, our finding that coinfection of latent genital *C. muridarum*-infected mice with *N. gonorrhoeae* did not result in increased vaginal PMNs, compared to *N. gonorrhoeae* single infection, in contrast to observations for acute genital *C. muridarum*/*N. gonorrhoeae*-coinfected mice versus *N. gonorrhoeae* singly infected mice (27), may be explained by a lack of increased vaginal *N. gonorrhoeae* infection/shedding in our setting.

Finally, our observed trend toward *reduced* vaginal PMNs in latent genital *C. muridarum*/*N. gonorrhoeae*-coinfected versus singly infected mice (*N. gonorrhoeae* $>$ *C. muridarum*+*N. gonorrhoeae* $>$ *C. muridarum*, both in vaginal tissues and vaginal smears representing luminal PMNs), contrasts previous *acute* genital *C. muridarum*/*N. gonorrhoeae* findings (27). This may support the recent finding that chlamydial PMN infection renders PMNs less responsive to *N. gonorrhoeae* (10). Chlamydial protease-like activity factor (CPAF) cleaved human PMN N-formyl peptide receptor 2 (FPR2), "paralyzing" the PMNs, and also inhibited murine PMNs (which express a FRP2 homologue) (10, 54). Thus, our findings may indicate that latent genital chlamydial infection may inhibit murine PMNs *in vivo*. This finding was not associated with modulation of *C. muridarum* or *N. gonorrhoeae* vaginal shedding/recovery or measures of tissue pathology in our study. However, we may speculate that a similar phenomenon in humans might reduce overall *N. gonorrhoeae* symptoms, perhaps hindering or delaying diagnosis or treatment and representing a source of potential transmission.

In summary, we show (i) that *N. gonorrhoeae* coinfection does not reactivate *C. muridarum* vaginal shedding from a state of *C. muridarum* genital latency; (ii) that latent chlamydial genital infection, unlike acute chlamydial genital infection as previously reported (27), does not increase recovery of viable vaginal *N. gonorrhoeae*; and (iii) that

latent genital *C. muridarum* infection may limit subsequent *N. gonorrhoeae*-dependent vaginal PMN induction, consistent with chlamydial inhibition of normal PMN function against *N. gonorrhoeae* (10). Our findings expand on previous findings to suggest differing effects of acute versus latent chlamydial genital infection on subsequent *N. gonorrhoeae* genital infection in mice. Continued evaluation of *Chlamydia*/*N. gonorrhoeae* interaction *in vivo*, including how such coinfections may impact the host, is important to inform our continued efforts toward the prevention and/or treatment of chlamydia and gonorrhea.

MATERIALS AND METHODS

Host cells and media. LLC-MK2 cells (LLC; rhesus monkey kidney cell line; provided by IZSLER, Brescia, Italy) were cultivated as previously described (28).

***C. muridarum* propagation and inoculum preparation.** *C. muridarum* Weiss strain (obtained from Kyle Ramsey, Midwestern University) was propagated in LLC cells, crude stocks were prepared, and IFU/mL were determined as previously described (28, 55, 56).

Mycoplasma testing. DNA extracted from cells and chlamydial stock was tested using a mycoplasma detection kit (VenorGeM OneStep; MB Minerva Biolabs, Germany) for conventional PCR according to the manufacturer's instructions. Cells and chlamydial stock used in this study were *Mycoplasma* negative.

***N. gonorrhoeae* propagation and inoculum preparation.** *N. gonorrhoeae* strain FA1090 (endocervical isolate, originally isolated in 1983 from a probable disseminated infection) (57) was provided by Magnus Unemo, School of Medical Sciences, Orebro University, and cultivated on commercially available chocolate agar (chocolate agar with Vitox; Thermo Fisher Scientific), as previously described (28). For inocula, *N. gonorrhoeae* colonies (18 to 24 h) were collected with a sterile nylon swab and suspended in sterile phosphate-buffered saline (PBS [pH 7.2], 1-L tablets; Canvax Biotech, Córdoba, Spain) to a McFarland density of approximately 2.0 to 3.0 (DEN-1B densitometer; Grant Instruments, Cambridgeshire, UK; 0.5-4.0 McFarland Standard Set, Pro Lab Diagnostics, Richmond Hill, Ontario, Canada). The suspended *N. gonorrhoeae* was then passed through a sterile 1.2-μm-pore size filter to remove aggregates, as previously described (33), and diluted with sterile PBS to a McFarland density of approximately 0.62 to 0.67, which we determined by trial to represent CFU/mL of $\sim 10^8$ CFU/mL. Inocula CFU/mL ranged from (1.7 to 2.2) $\times$ 10^6, as determined by dilution of the filtered suspensions in sterile 0.05% saponin (Sigma-Aldrich/MilliporeSigma)/PBS cultured on chocolate agar for 18 to 24 h with bacterial colonies counted on a stereo microscope (M3; Wild Heerbrugg AG, Heerbrugg, Switzerland).

Coinfection protocol. Female BALB/c mice, 6 weeks of age, were purchased from Janvier Labs (Le Genest-Saint-Isle, France), housed five mice per cage with 12-h light/dark cycle, regulated temperature/humidity, with *ad libitum* access to standard mouse feed and water, and allowed to acclimate for 12 days with the inclusion of a tunnel for both enrichment and mouse handling as refinement to reduce stress (50, 51). The experimental design is shown in Fig. 1; the day of the experiment is noted as "day 0" to "day 36." The study comprised 90 mice evaluated in three independent experiments carried out over 9 months between November 2020 and July 2021; (see File S1). Mice were divided into four groups: mice coinfected with *C. muridarum* and *N. gonorrhoeae* (*C. muridarum*+*N. gonorrhoeae*), mice infected with either pathogen alone (*C. muridarum* or *N. gonorrhoeae*), and mice mock infected with SPG (sucrose phosphate glutamate)/PBS alone as a control (UN). Mice inoculated with *C. muridarum* (the *C. muridarum* and *C. muridarum*+*N. gonorrhoeae* groups) were vaginally inoculated for three consecutive days with 10^6 IFU of *C. muridarum* in 10 μL of SPG (sucrose phosphate glutamate buffer, 218 mM sucrose; Sigma-Aldrich/Millipore Sigma); 3.76 mM KH_2PO_4 (Sigma-Aldrich/Millipore Sigma), 7.1 mM K_2HPO_4 (Sigma-Aldrich/Millipore Sigma), and 5 mM GlutaMAX (Gibco, Thermo Fisher Scientific) on days 0, 1, and 2 to increase the likelihood that mice were in the diestrus stage of the reproductive cycle, which is critical for a robust chlamydial infection (30). Mice not inoculated with *C. muridarum* (the UN and *N. gonorrhoeae* groups) were vaginally similarly inoculated with SPG alone.

On experiment day 25, 23 days after chlamydial infection, during chlamydial latency, *N. gonorrhoeae* infection was carried out essentially as described previously (27, 33). Vaginal smear slides were prepared from all mice and stained with Hematek Stain Pak 4481 modified Wright stain (Siemens AG; Munich, Germany). Mice with predominantly vaginal neutrophils and nucleated epithelial cells, rather than cornified epithelial cells (as determined by cell morphology [33]), were considered to be in diestrus, while mice with few cells, visible bacteria and mucus were considered to be in anestrus. Mice in diestrus or anestrus were treated with subcutaneous injections of Premarin (0.5 mg), a conjugated estrogen, once per day mornings; (Sigma) on days 23, 25, and 27 to promote gonococcal infection and prolonged colonization. Intraperitoneal injections of vancomycin hydrochloride (0.6 mg) and streptomycin sulfate (2.4 mg) were given to all mice twice daily, mornings and afternoons, for 4 days (from days 23 to 27; on day 23, the vancomycin/streptomycin was administered only once in the afternoon) to control growth of commensal flora, caused by Premarin treatment. Moreover, from days 28 to 36, 5 g/L streptomycin sulfate dissolved in autoclaved tap water was provided *ad libitum* to all groups to continue to help control commensal flora overgrowth. Mice not in diestrus or anestrus on day 23 were sacrificed that day.

On day 25, $\sim$4 h after the second dose of Premarin, mice were inoculated vaginally with either 20 μL of PBS (UN and *C. muridarum* groups) or with *N. gonorrhoeae* in 20 μL of PBS [range, (1.7 to 2.2) $\times$ 10^6 CFU; *N. gonorrhoeae* and *C. muridarum*+*N. gonorrhoeae* groups]. Vaginal swabs, collected

every 2 to 3 days, until the end of experiment, were used to determine first vaginal chlamydial shedding of live EBs (indicative of active *C. muridarum* infection) and chlamydial latency (lack of vaginal shedding of live EBs) by titer assay (days 4 to 24). After *N. gonorrhoeae* infection (days 27 to 36), both *C. muridarum* shedding and recovery of viable *N. gonorrhoeae* from vaginal swabs were determined by titration. At day 36, all mice were sacrificed, necropsy was performed, and tissue samples were collected and stored for histopathology and molecular analysis. In addition, at sacrifice, rectal swabs were collected for *C. muridarum* shedding determination, and vaginal smear slides were prepared for PMN analysis. Researchers were not blinded to mouse procedures or sample preparations, due to technical requirements. However, all microscopic analyses were carried out in a blinded manner, with the exception of pathological and immunohistochemistry analyses of tissues.

Sample collection. (i) Vaginal and rectal swabs and vaginal smears. Mice were vaginally or rectally swabbed with a PBS-soaked swab (Ultra Mini Flocked Swab, Copan 516CS01; Copan Italia, Brescia, Italy) inserted into the vaginal canal or rectum and rotated gently. Swabs were collected into 2-mL tubes containing 1 mL of sterile SPG and three sterile glass beads. Swab tubes were vortexed for 10 s and processed for recovery of viable *N. gonorrhoeae* (vaginal swabs only, see below) was carried out prior to snap-freezing on dry ice and storage at −80°C. Vaginal smears were collected by inserting a PBS-moistened swab into the vagina, rotating gently as for vaginal swabs, and smearing the collected material on standard glass microscopy slides.

(ii) Necropsy, tissue sampling, and pathology scoring. Necropsy included observation of gross genital tract macroscopic findings. The genital tract (ovary, oviduct, uterus, cervix, and vagina) and the intestinal tract (duodenum, jejunum, ileum, cecum, colon, and rectum) were collected and stored in 4% buffered formaldehyde for 24 h, followed by embedding in paraffin according to routine procedure. From each formalin-fixed and paraffin-embedded (FFPE) block, 2-μm sections were cut and stained with hematoxylin and eosin (H&E), followed by microscopic evaluation of complete longitudinal sections by a board-certified pathologist.

Histologic evaluation focused on the oviduct, cervix, and vagina. For the oviduct assessment, available cross sections on the slide were counted and the number of dilated versus total number of cross sections was calculated. In addition, the presence of inflammation (yes/no) was recorded, and the type of inflammatory infiltrate was described in a qualitative manner. For the cervix and the vagina, the stage of epithelial differentiation was recorded, and the presence of neutrophils was assessed semiquantitatively (luminal versus epithelial, mild, moderate, and severe).

Quantification of viable chlamydial shedding by titration and immunofluorescence microscopy. Quantification of vaginal or rectal viable *C. muridarum* shedding was determined by titration in LLC cells. Briefly, after overnight LLC culture, triplicate serial dilutions of thawed, vortexed swab samples were used to infect LLC as previously described (28). Inocula were replaced with growth medium supplemented with cycloheximide (Sigma) at a 1.5-μg/mL final concentration and antibiotics (amphotericin B [Gibco] at a 1.3-μg/mL final concentration, vancomycin hydrochloride [Sigma] at a 100-μg/mL final concentration, and gentamicin [Gibco] at a 10-μg/mL final concentration), and plates were incubated for 24 h at 37°C and 5% CO_2. The cells were then fixed, and *C. muridarum* inclusions were immunostained as previously reported (28) Chlamydial inclusions were counted at a $\times$100 to $\times$200 magnification, and IFU/swab calculation was performed (limit of detection was 5 IFU/swab, at 200 μL of total swab volume assayed in the least diluted triplicate titration wells; Eclipse TiU; Nikon Instruments, Inc., Melville, NY). Vaginal swabs across all experiments for all groups (UN and *N. gonorrhoeae*) not *C. muridarum* inoculated were assayed as described, except 300 μL of total swab volume was assayed undiluted in triplicate wells (100 μL/well), to confirm the absence of *C. muridarum* cross-contamination.

Quantification of recovery of viable *N. gonorrhoeae* by titration. Swab samples vortexed for 5 to 10 s were added, in triplicate, to 0.05% saponin-PBS, in 96-well U-bottom plates, and 1:3 serial dilutions (eight dilutions) were performed; then, 5 μL of each dilution well and three 34-μL replicates of undiluted sample were plated to commercial selective agar plates (*Neisseria* Selective Medium PLUS; Thermo Scientific) with antibiotic selection (vancomycin, colistin, amphotericin B, and trimethoprim), followed by incubation for 24 to 48 h at 37°C and 5% CO_2. *N. gonorrhoeae* colonies were counted on a stereomicroscope (M3; Wild Heerbrugg AG), and *N. gonorrhoeae* shedding (CFU/swab) was calculated (the limit of detection was 10 CFU/swab, at 100 μL of undiluted sample assayed). Vaginal swabs for all groups across all experiments (UN and *C. muridarum*) not *N. gonorrhoeae* inoculated were assayed as described, except that 50 μL of total swab volume was assayed to confirm the absence of *N. gonorrhoeae* cross-contamination.

DNA extraction and *Chlamydiaceae* real-time PCR. DNA extracted from 150 μL of vaginal swab or rectal swab samples, using the QIAamp DNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations, was eluted in a 50-μL final volume. DNA extracted from tissue samples (20-μm sections of FFPE blocks as previously described [58]) was eluted in a 50-μL final volume. *C. muridarum* positivity (expressed in units of genome copies/swab) was determined by quantitative real-time PCR (qPCR) based on the *Chlamydiaceae* family-specific 23S rRNA gene, as previously described (58, 59). All samples were tested in duplicate at 7.5 μL of total swab volume assayed as 2.5 μL of DNA per qPCR (limit of detection, $\sim 10^2$ genome copies), and a cycle threshold (C_T) value of $<$38 was considered to be positive for *C. muridarum* and used to calculate chlamydial genome copies/rectal swab, while vaginal swab and tissue samples were scored only as negative or positive; a positive control comprising a 7-fold *C. abortus* DNA dilution series and a negative control (water instead of template DNA) were included in each run (60).

CM and *N. gonorrhoeae* detection by immunohistochemistry. Complete longitudinal genital tract sections of all mice (n = 89) were immunolabeled for *C. muridarum* and *N. gonorrhoeae*. Intestinal tract tissue sections (consisting of one cross section each from the duodenum, jejunum, ileum, cecum, colon,

and rectum) from the *C. muridarum* and *C. muridarum*+*N. gonorrhoeae* groups ($n = 49$) were immunolabeled for *C. muridarum*, and those from the *N. gonorrhoeae* and *C. muridarum*+*N. gonorrhoeae* groups ($n = 59$) were immunolabeled for *N. gonorrhoeae*. Primary antibodies included a *Chlamydiaceae* family-specific rabbit polyclonal antibody LPS/MOMP antibody (Cygnus Technologies, Inc., Southport, NC) at a 1:1,000 dilution for *C. muridarum* antigen detection and a rabbit anti-*N. gonorrhoeae* polyclonal antibody (Abnova, Taipeh, Tawain) at a 1:1,000 dilution for *N. gonorrhoeae*. Antigen retrieval consisted of pressure cooking (98°C) in citrate buffer (Dako/Agilent, Santa Clara, CA) for 20 min for *Chlamydia* and treatment with FastEnzyme (Zytomed Systems GmbH, Bargteheide, Germany) for 10 min at room temperature for *Neisseria*, respectively. After incubation with the primary antibodies for 1 h at room temperature, the endogenous peroxidase activity (Dako Agilent) was inhibited for 10 min at room temperature. Detection was performed with Envision + System HRP Rabbit (Dako Agilent) for a 30-min incubation at room temperature, using the substrate 3-amino-9-ethylcarbazole (AEC)-peroxidase with a hematoxylin counterstain. For the positive controls, lung tissue from a *C. pneumoniae*-infected mouse (*Chlamydia* control, kindly provided by Bernhard Kaltenboeck) and a cell pellet array, including *N. gonorrhoeae*-infected LLC-MK2 and HeLa cells, was used.

PMN semiquantification. Vaginal smear slides prepared at the end of the experiment (day 36) were stained to semiquantify the PMNs. Briefly, smears were stained with Hematek stain, evaluated (as described above), and categorized as having (i) no/rare PMNs (consistent with estrus as induced by estradiol/estrogen) versus (ii) few/more PMNs (consistent with PMN influx into the vaginal lumen).

Statistical analysis. Sample size for the primary analyses of *C. muridarum* and *N. gonorrhoeae* vaginal shedding was estimated based on simulated power analysis under design of two-way analysis of variance with a repeated-measures model. A final sample size of $n = 14$ mice per group (except for the mock-treated group, which was not directly compared, and considering mice dropped out due to estrous stage) was determined to ensure the probability of 90% that the expected power is at least 0.80. Experimental units were completely randomized to experimental groups in each of the independent experiments.

The significance of any observed differences in viable *C. muridarum* and *N. gonorrhoeae* vaginal shedding between experimental groups was evaluated using a linear mixed-effect model, taking into account repeated measures over time of the same mouse and excluding any mice for which viable *C. muridarum* shedding or recovery of viable *N. gonorrhoeae* could not be detected in any of the 12 vaginal swabs collected. Analysis was performed on $\log_{10}$-transformed IFU/swab and CFU/swab data, with values of 1 IFU or CFU below the limit of detection (i.e., 4 and 9, respectively) assigned to individual swab samples with no *C. muridarum* or *N. gonorrhoeae* detected.

The significance of any observed differences was evaluated for five pathology outcomes (oviduct dilation ratio [dilated/total], oviduct dilation score [none, mild, moderate, or severe], oviduct inflammation [yes versus no], as well as cervical and vaginal infiltration with PMNs graded from 0 to 3 [none, mild, moderate, or severe]). Early-sacrifice mice were evaluated as two groups: mock infected (the UN and *N. gonorrhoeae* groups) or *C. muridarum* infected (the *C. muridarum* and *C. muridarum*+*N. gonorrhoeae* groups). Late-sacrifice mice were evaluated as four groups: UN, *C. muridarum*, *N. gonorrhoeae*, and *C. muridarum*+*N. gonorrhoeae*. Mice with tissue sections missing were excluded from pathology analyses. For the first pathology outcome, the oviduct dilation ratio, which is a continuous variable, we applied the *t* test. For the other four pathology outcomes which are either ordinal or categorical variables, we applied the Fisher exact test.

The significance of any observed differences in live *C. muridarum* rectal shedding or *C. muridarum* genome copies/swab between experimental groups were evaluated using the *t* test; analysis was performed on $\log_{10}$-transformed IFU/swab and, with values of 1 IFU below the limit of detection (i.e., 4) assigned to individual swab samples with no *C. muridarum* detected. The proportion of mice *C. muridarum* rectal swabs determined to be positive by titer and by *Chlamydiaceae* qPCR, respectively, was compared between the *C. muridarum* and *C. muridarum*+*N. gonorrhoeae* groups, and the proportion of mice *N. gonorrhoeae* IHC positive in the genital tract was compared between the *N. gonorrhoeae* and *C. muridarum*+*N. gonorrhoeae* groups; for these comparisons we applied the Fisher exact test.

Statistical analysis was performed using R statistical software (https://www.R-project.org/).

Animal use. Unless otherwise stated (as in File S3), animal experiments were conducted in the Laboratory Animal Services Center (LASC) at University of Zurich (BSL-2) and previously approved by Cantonal Veterinarian's Office of Zurich (license 018/2020). Refinements were made to minimize animal stress.

Data availability. The original contributions presented are included within the article and in the supplemental material. Further inquiries can be directed to the corresponding author.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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