AMERICAN SOCIETY FOR MICROBIOLOGY | Infection and Immunity

Microbial Pathogenesis | Full-Length Text

Neisseria gonorrhoeae drives *Chlamydia trachomatis* into a persistence-like state during *in vitro* co-infection

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ABSTRACT *Chlamydia trachomatis* and *Neisseria gonorrhoeae* are the most prevalent bacterial sexually transmitted infections (STIs) globally. Despite frequent co-infections in patients, few studies have investigated how mono-infections may differ from co-infections. We hypothesized that a symbiotic relationship between the pathogens could account for the high rates of clinical co-infection. During *in vitro* co-infection, we observed an unexpected phenotype where the *C. trachomatis* developmental cycle was impaired by *N. gonorrhoeae*. *C. trachomatis* is an obligate intracellular pathogen with a unique biphasic developmental cycle progressing from infectious elementary bodies (EB) to replicative reticulate bodies (RB), and back. After 12 hours of co-infection, we observed fewer EBs than in a mono-infection. Chlamydial genome copy number remained equivalent between mono- and co-infections. This is a hallmark of Chlamydial persistence. Chlamydial persistence alters inclusion morphology but varies depending on the stimulus/stress. We observed larger, but fewer, *Chlamydia* during co-infection. Tryptophan depletion can induce Chlamydial persistence, but tryptophan supplementation did not reverse the co-infection phenotype. Only viable and actively growing *N. gonorrhoeae* produced the inhibition phenotype in *C. trachomatis*. Piliated *N. gonorrhoeae* had the strongest effect on *C. trachomatis*, but hyperpiliated or non-piliated *N. gonorrhoeae* still produced the phenotype. EB development was modestly impaired when *N. gonorrhoeae* were grown in transwells above the infected monolayer. *C. trachomatis* serovar L2 was not impaired during co-infection. Chlamydial impairment could be due to cytoskeletal or osmotic stress caused by an as-yet-undefined mechanism. We conclude that *N. gonorrhoeae* induces a persistence-like state in *C. trachomatis* that is serovar dependent.

KEYWORDS *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *in vitro* co-infection, persistence, serovar specificity

Chlamydia trachomatis and *Neisseria gonorrhoeae* are, respectively, the number one and number two most prevalent bacterial sexually transmitted infections internationally (1, 2). In adults around the world, *C. trachomatis* is estimated to cause 130 million new infections and *N. gonorrhoeae* is estimated to cause 87 million new infections, annually. Both infections are frequently asymptomatic, especially in women, with primary infection beginning at the mucosal epithelium of the genital tract (3). Rarely, the infections can disseminate beyond the genital tract (4, 5). Women are particularly at risk of an upper genital tract infection leading to pelvic inflammatory disease (PID), ectopic pregnancy, and infertility due to chronic inflammation induced by both bacterial pathogens (6). While antibiotic resistance has not been reported for *Chlamydia* infections, *N. gonorrhoeae* first developed resistance to antibiotic therapy in the 1950s and continues to evolve resistance to newly introduced drugs (7). This makes treatment for co-infections more of a challenge as drugs effective against *C. trachomatis* may not always be effective against *N. gonorrhoeae*.

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The authors declare no conflict of interest.

In memory of Dr. Josephine Clark-Curtiss, whose advice and expertise helped this project reach fruition.

Received 9 May 2023
Accepted 5 November 2023
Published 28 November 2023

C. trachomatis is an obligate intracellular pathogen that undergoes a unique developmental cycle beginning with its infectious but not replicative form—the elementary body (EB) (8, 9). After invasion into a susceptible host cell and establishment of an inclusion within the host cell cytoplasm, the EB differentiates to become a reticulate body (RB) which is non-infectious. The RB divides by binary fission causing the inclusion to expand as the population of bacteria increases, until asynchronous differentiation begins and the RBs transition to EBs. At the end of the developmental cycle, the inclusion lyses or extrudes from the host cell. EBs can be detected as early as 18 hours post-infection (hpi) for *C. trachomatis* and the developmental cycle typically concludes at 48 hpi in *in vitro* settings (10). When the environment for the bacteria within the inclusion becomes sub-optimal, including but not limited to iron deprivation, nutrient deprivation, antibiotic exposure, or interferon gamma-induced tryptophan starvation, the RBs transition into aberrant bodies (ABs) and stop dividing while remaining viable (11). This is called "persistence" and enables long-term survival for *C. trachomatis* inside the parasitized host cell. This state is reversible, and the bacteria return to complete the developmental cycle once the stressing agent is gone. Recently, a comprehensive study characterized the persistent phenotypes and showed that different persistence-inducing conditions produce different Chlamydial phenotypes including loss of homotypic fusion (the ability of multiple inclusions within a single cell to fuse), peptidoglycan synthesis arrest, absence of secreted mid- and late-stage bacterial effectors and/or loss of cellular actin recruitment to the inclusion membrane (12).

N. gonorrhoeae is a facultative intracellular pathogen. The invasion of epithelial cells is dependent on the expression of both type 4 pili (T4P) and opacity-associated proteins (Opa) (13). Invasion leads to transcytosis through the epithelial cells (14). In symptomatic gonorrhea, gonococci are usually observed inside and attached to neutrophils which make up the characteristic purulent discharge. Extensive research has demonstrated the ability of *N. gonorrhoeae* to survive and replicate inside neutrophils and impair their antimicrobial activity (15). In *N. gonorrhoeae*, T4P are retractable extracellular structures, with most of the pilus structure comprised of PilE, and PilT the ATPase responsible for retracting the pilus. Alternating extrusion and retraction of T4P enables the characteristic gonococcal "twitching motility." During retraction, the T4P exerts a measurable physical force on the plasma membrane of eucaryotic cells and is known to disrupt cell signaling of epithelial cells (16–18).

The extent of *C. trachomatis–N. gonorrhoeae* co-infections is difficult to accurately quantify due to the frequent asymptomatic nature of these infections. Incidence is generally estimated at 50%–70%. However, across seven studies, women with *N. gonorrhoeae* are most likely to be co-infected with *C. trachomatis* 17.6%–57.9%, whereas women with *C. trachomatis* are co-infected with *N. gonorrhoeae* in 2.1%–17.2% of cases (19–25). While both organisms are capable of ascension and causing PID, there does not seem to be evidence that co-infections specifically produce a higher proportion of acute PID (26).

Laboratory studies of *C. trachomatis–N. gonorrhoeae* co-infection in *in vitro* epithelial cells have only recently been reported (27). Onorini et al. examined the effect of gonococcal infection of HeLa cells prior to the *C. trachomatis* serovar E challenge. They observed that during co-infection, the *C. trachomatis* inclusions are reduced to 2% of their size when compared to a *C. trachomatis* mono-infection. *N. gonorrhoeae* pre-infection also reduced the quantity of inclusions observed, by up to 40%. The number of EBs produced during co-infective conditions decreased approximately 10-fold. This phenotype was not due to acidification of the medium nor nutrient deprivation from bacterial competition. The authors modified their experimental timeline and added *N. gonorrhoeae* after the *C. trachomatis* infection and allowed the bacteria to grow in parallel for 24 hours. As before, they observed reduced inclusion size and quantity when *N. gonorrhoeae* is added after *C. trachomatis* infection. Co-infection in human neutrophils

has also been examined and found that *C. trachomatis* serovar L2 infection of neutrophils reduced their ability to kill *N. gonorrhoeae* without loss of neutrophil health (28).

To date, there are only two published reports that detail infection of mice with both *Chlamydia* and *N. gonorrhoeae* (29, 30). Both studies utilized the mouse pathogen *C. muridarum* as a surrogate for *C. trachomatis*. Vonck et al. pre-infected mice with *C. muridarum* and then challenged the mice with *N. gonorrhoeae* (29). In this study, co-infection did not increase *Chlamydial* titer but did increase *N. gonorrhoeae* shedding. In the same study, this *in vivo* result was not recapitulated by *in vitro* co-infection, as co-infection did not increase adhesion or invasion of *N. gonorrhoeae* to the infected cultured cells. While *C. muridarum*-specific antibody titers were not increased by co-infection, neutrophil influx was increased during co-infection. More recently, Onorini et al. established a latent *C. muridarum* infection in mice before challenge with *N. gonorrhoeae* at day 24 hpi (30). After 12 days of co-infective growth, *C. muridarum* titer was not increased by the gonococcal presence.

In this study, we aimed to identify whether a mid-cycle *C. trachomatis* infection is affected by a subsequent gonococcal challenge and, if so, how one or both bacterial species are affected. We found that while growth (as measured by genome copy number) of a urogenital serovar of *C. trachomatis* (serovar D) was unaffected, the titer of the infectious, fully differentiated bacteria was reduced 100-fold. We found that the phenotype was not produced when *N. gonorrhoeae* were killed by isopropanol or inhibited by the addition of gentamycin; could not be restored by tryptophan nor indole supplementation; that the phenotype was reversible; and that a partial phenotype was produced when gonococci were grown separated from the monolayer by transwells. When we challenged the Chlamydial infection with non-piliated or hyperpiliated *N. gonorrhoeae*, we saw a partial inhibition of EB development compared to a mono-infection. This suggested that while the action of T4P was responsible for the persistent-like phenotype in *C. trachomatis*, they are not the sole cause for the Chlamydial phenotype we observed during *C. trachomatis–N. gonorrhoeae* co-infection. Interestingly, the phenotype was biovar dependent as we did not observe the same inhibition when we used a lymphogranuloma venereum (LGV) serovar *C. trachomatis* L2. We considered that a major difference between *C. trachomatis* L2 and D serovars is the presence of the putative *Chlamydia* cytotoxin (31). We used *C. muridarum* (which has a complete cytotoxin locus) in the co-infection but did not observe a 100-fold or greater decrease in EB production as we anticipated. This result does not suggest that the cytotoxin alone is the cause behind the co-infection *C. trachomatis* phenotype as we hypothesized.

Co-infections are an important aspect of sexually transmitted infection, and it is critical that we know how they differ from mono-infections. Our observations provide insights into how co-infections of *C. trachomatis* and *N. gonorrhoeae* may be affected by current and future treatments.

RESULTS

N. gonorrhoeae reduces the number of *chlamydial* EBs after 12 hours of co-infection

To probe the interaction between *C. trachomatis* and *N. gonorrhoeae* in a cell culture model, we pre-infected sub-confluent HeLa monolayers with *C. trachomatis* serovar D and then challenged with *N. gonorrhoeae* at 12 hpi. The gonococcal challenge continued for 12 hours then all bacteria were enumerated. At this point in the *Chlamydial* developmental cycle, 24 hpi, we expect inclusions to contain mostly RBs and a small quantity of EBs, as EBs are detectable at 18 hpi (10). Figure 1A illustrates the timeline of the co-infection. When comparing the final number of *N. gonorrhoeae* attached to the monolayer, the colony forming units (CFU)/mL titer between mono-infection and co-infections showed no difference (Fig. 1B). We saw no difference between *C. trachomatis* genome copy (GC) number (used as a proxy for all *C. trachomatis* developmental forms) when comparing *C. trachomatis* quantity between mono- and co-infections (Fig. 1C). However, when we measured the titer of infectious EBs by counting the number of

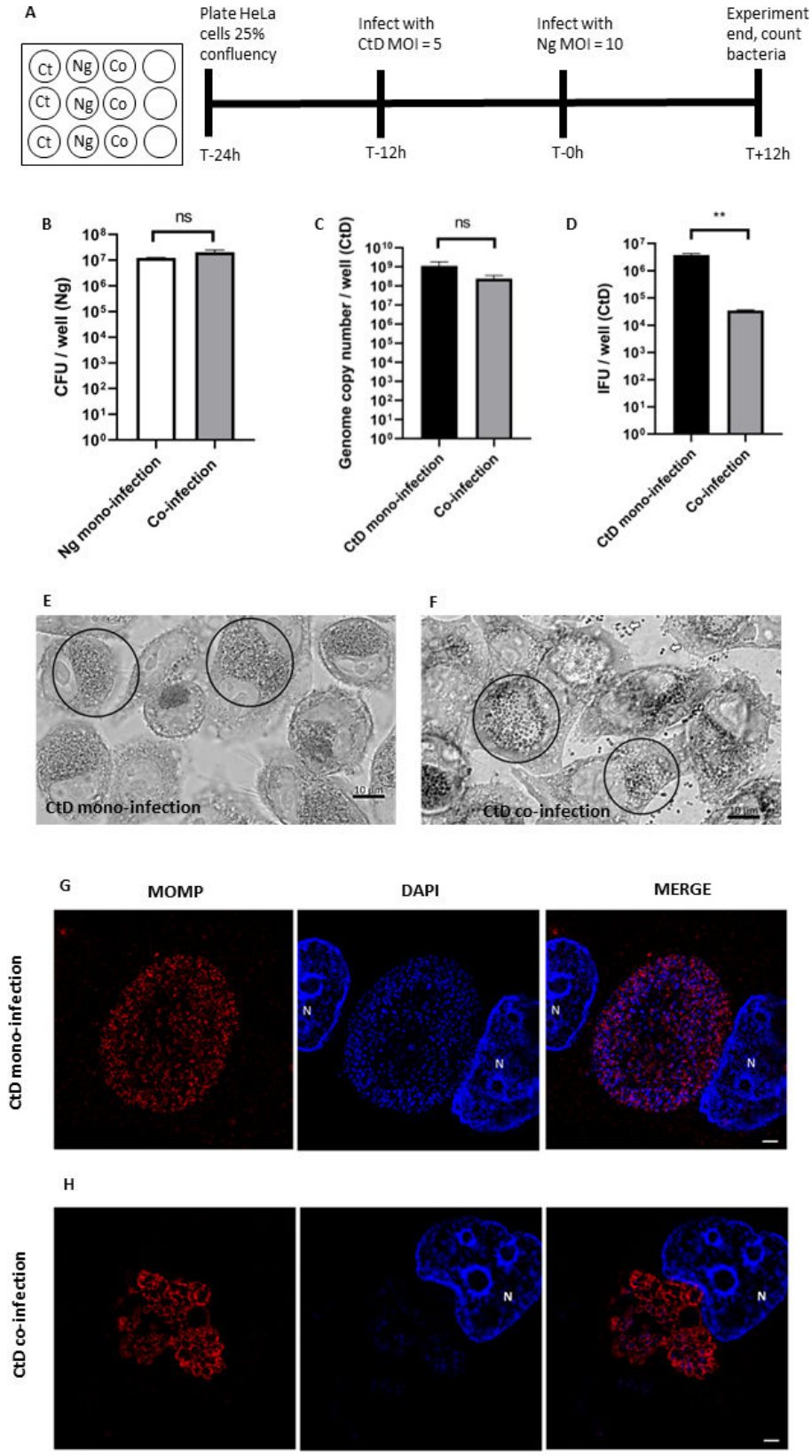

FIG 1 *C. trachomatis* EB titer is reduced after co-infection with *N. gonorrhoeae*. (A) Infections were carried out as described in Materials and Methods. (B) *N. gonorrhoeae* (Ng) CFU/mL after 12 hours of co-infection with or without *C. trachomatis* D. (C) Quantitative PCR measurement of genome copy number for all forms of *C. trachomatis* (CtD) (EBs, RBs, and ABs)

(Continued on next page)

FIG 1 (Continued)

after 24 hours of growth in HeLa cells including 12 hours of co-infection with or without *N. gonorrhoeae*. (D) Quantitative immunofluorescence analysis of *C. trachomatis* D (CtD) EB titer after 24 hours of growth in HeLa cells including 12 hours of co-infection with or without *N. gonorrhoeae*. Statistical analyses by Student's *t*-test. Error bars are SEM. All experiments were performed in triplicate and repeated a total of three times. Differential interference contrast microscopy (DCIM) microscopy images of Giemsa stained (E) *C. trachomatis* mono-infection and (F) *C. trachomatis–N. gonorrhoeae* co-infection. Cells were fixed with 4% paraformaldehyde and stained with Giemsa stain then imaged at 1,000×. *C. trachomatis* inclusions are circled. In (F), *N. gonorrhoeae* cocci and diplococci can also be seen exterior to the cells (open arrows). Structured illumination microscopy (SIM) images of (G) *C. trachomatis* mono-infection and (H) *C. trachomatis–N. gonorrhoeae* co-infection. Chlamydial cells are larger after 12 hours of co-infection and aberrant bodies (ABs) are visible. Cells were fixed and permeabilized with methanol and 0.5% Triton X-100. *Chlamydia* appears red and DNA appears blue. Host cell nuclei are noted with "N." White scale bars are 2 µm.

inclusions using immunofluorescence assay (IFA), there was a stark difference between mono- and co-infections and the EB titer was 100-fold fewer in a co-infection (Fig. 1D).

N. gonorrhoeae changes chlamydial inclusion morphology

We noticed the addition of *N. gonorrhoeae* impacted the appearance of the Chlamydial inclusions at 12 hpci. After fixing with 4% paraformaldehyde and staining with Giemsa, we were able to see the bacteria within the inclusions during co-infection. At 100×, the bacteria within the inclusions look enlarged and more sparsely distributed than the bacteria within a mono-infection (Fig. 1E and F). In addition, we used structured illumination microscopy to examine the morphology of the bacterial cells within the inclusions. Individual *C. trachomatis* cells are enlarged, consistent with an aberrant body (AB) phenotype (Fig. 1G and H)

Killed or inhibited *N. gonorrhoeae* do not affect *chlamydial* development

After our initial observations of a dramatic decrease in *Chlamydial* EB titer following *C. trachomatis–N. gonorrhoeae* co-infection, we asked whether this could be due to the presence of viable *N. gonorrhoeae*, or if it was because of nutrient depletion due to gonococcal growth. To answer this question, the co-infection experiment was repeated; however, before infection, the *N. gonorrhoeae* inocula were centrifuged and resuspended in 70% isopropanol or a medium containing gentamycin. After 10 minute of incubation in 70% isopropanol, the bacteria were re-centrifuged and resuspended in a fresh medium. Viability counts of the isopropanol-treated *N. gonorrhoeae* inoculum indicated that all the bacteria were effectively killed (Fig. 2A). Viability counts for gentamycin-treated *N. gonorrhoeae* inoculum indicated that the bacteria were alive when added to the co-infection but inhibited from growth during the duration of the experiment. The pre-treated inocula were added to *C. trachomatis*-infected cells as described above and the co-infection incubated for 12 hours. When pre-killed or growth-inhibited *N. gonorrhoeae* were used in the co-infection, the EB titer was restored to that of a mono-infection but the GC number for total *C. trachomatis* was not different between any of the conditions (Fig. 2B and C). This observation suggests that it is critical for *N. gonorrhoeae* to be actively growing with *C. trachomatis*-infected HeLa cells to decrease the *Chlamydial* EB titer.

The inhibition phenotype is reversible

To fulfill all the requirements for a truly persistent phenotype, *Chlamydial* growth must be restored when the stressing agent is removed. We performed the standard co-infection but at 12 hours post-co-infection (hpci), the supplemented RPMI medium containing *N. gonorrhoeae* was removed, and the monolayer was carefully washed with PBS to detach loosely adherent *N. gonorrhoeae* without detaching any fragile aberrant inclusion-containing HeLa cells. The medium was replaced with fresh supplemented RPMI ± 20 µg/mL gentamycin. The co-infection was allowed to continue for an additional

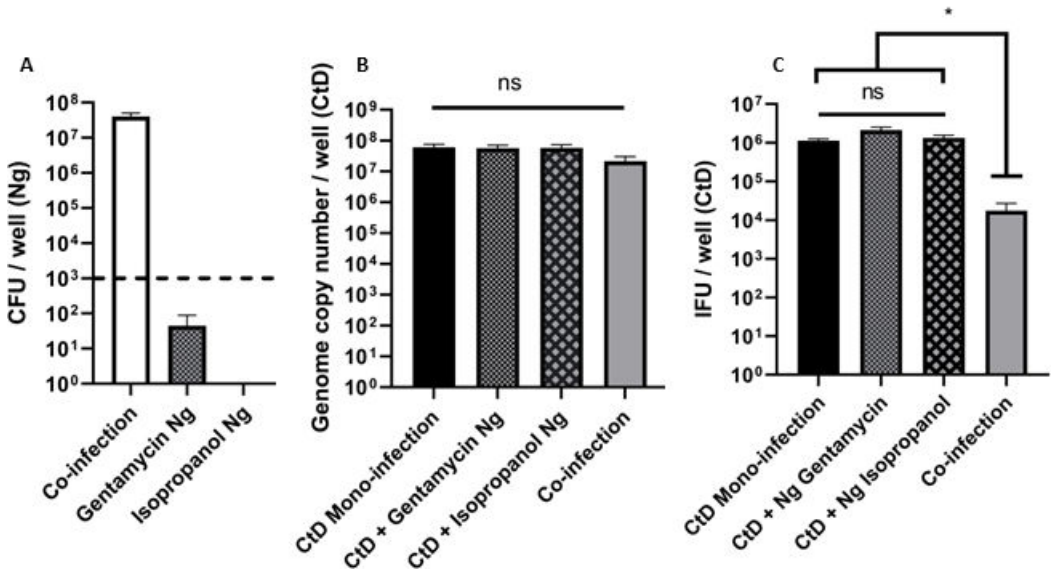

FIG 2 Dead or inactivated *N. gonorrhoeae* do not inhibit *C. trachomatis* EB titer during co-infection. (A) *N. gonorrhoeae* (Ng) CFU/mL after 12 hours of co-infection. The dotted line denotes the limit of detection for this experiment. (B) Quantitative PCR analysis of genome copy number for *C. trachomatis* (EBs, RBs, and ABs) after 24 hours of growth in HeLa cells including 12 hours of co-infection with treated or untreated *N. gonorrhoeae.* (C) Quantitative immunofluorescence analysis of *C. trachomatis* D (CtD) EB titer after 24 hours of growth in HeLa cells including 12 hours of co-infection with treated or untreated *N. gonorrhoeae*. (C). Statistical analysis for data (C) is by one-way ANOVA $P = 0.0014$, with Tukey's repeated measures for the post hoc test, $^*P < .05$. Error bars are SEM and each experiment was performed in triplicate, three or four times.

12 hours and the experiment ended at 24 hpci. Figure S1 shows the modified co-infection timeline. All the bacteria were enumerated as for previous experiments. Figure 3A shows that the addition of gentamycin prevented regrowth of *N. gonorrhoeae*. The dotted line represents the limit of detection in this experiment. As with previous experiments, the GC number for *C. trachomatis* was unchanged between all samples (Fig. 3B). The EB titer for the *C. trachomatis–N. gonorrhoeae* gentamycin-treated co-infection was restored to an IFU/well count equivalent to the *C. trachomatis* mono-infection (Fig. 3C). We did not expect to see a partial recovery of EB titer in the absence of gentamycin (Fig. 3C, fourth bar). We hypothesize that this could be due to the medium change at 12 hpci which reduced the *N. gonorrhoeae* titer in the co-infection, replaced lost nutrients, and/or removed a secreted gonococcal molecule causing the chlamydial phenotype. During the following 12 hours of co-infection, *C. trachomatis* returned to its normal developmental cycle and produced EBs. The decrease between co-infections with and without gentamycin is not significantly different when analyzed by one-way ANOVA and Tukey's post hoc test. The restoration of *C. trachomatis* EB titer in a co-infection with gentamycin indicated that when the non-adherent *N. gonorrhoeae* were removed and residual adherent *N. gonorrhoeae* prevented from regrowth, *C. trachomatis* re-entered its normal developmental cycle and started producing EBs again. This result reinforces the conclusion that this phenotype could be a persistent state.

C. trachomatis EB titer is reduced during non-contact co-culture with *N. gonorrhoeae*

To further investigate the inhibitory effect of *N. gonorrhoeae* conditioned medium, we repeated the basic co-infection experiment (Fig. 1) but used 0.4 µm transwells to keep *N. gonorrhoeae* physically separated above the *C. trachomatis*-infected monolayer. *N. gonorrhoeae* are 0.5–1.0 µm in size due to their diplococcal morphology and cannot traverse these transwells. We hypothesized that we would see a decreased EB titer in the co-infected wells when *C. trachomatis* and *N. gonorrhoeae* shared the same culture

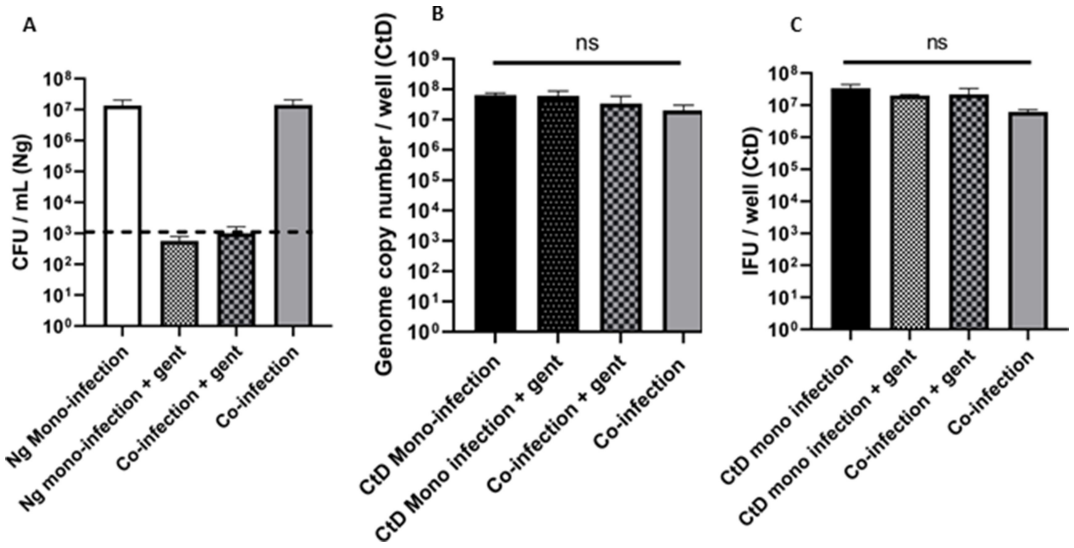

FIG 3 Removal of *N. gonorrhoeae* conditioned medium allows recovery of *C. trachomatis* EB titer. (A) *N. gonorrhoeae* (Ng) CFU/mL after 12 hours of co-infection and then 12 hours of ±gentamycin. The dotted line denotes the limit of detection for this experiment. (B) Quantitative PCR analysis of genome copy number for *C. trachomatis* D (EBs, RBs, and ABs) after 36 hours of growth in HeLa cells including 12 hours of co-infection ± *N. gonorrhoeae* and then 12 hours of ±gentamycin. (C) Quantitative immunofluorescence analysis of *C. trachomatis* D (CtD) EB titer after 36 hours of growth in HeLa cells including 12 hours of co-infection with or without *N. gonorrhoeae* and then 12 hours of ±gentamycin. Samples are not significantly different by one-way ANOVA or by Tukey's modified post hoc test. Error bars are SEM. Experiments were performed three times in triplicate.

medium. As seen previously, the gonococci grew to >10^8 CFU/mL in each of the three biological replicates (Fig. 4A) and the Chlamydial genome copy per well did not change significantly between mono- and co-cultures (Fig. 4B). We found that the EB titer for *C. trachomatis* was reduced approximately 10-fold which is a significant difference though not as pronounced as the 100-fold reduction when *N. gonorrhoeae* are grown in intimate contact with the eucaryotic cell plasma membrane (Fig. 4C). This indicated that merely sharing the nutrient pool was sufficient to produce a persistence-like response in *C. trachomatis* during *C. trachomatis–N. gonorrhoeae* co-culture.

Non-piliated *N. gonorrhoeae* decrease *C. trachomatis* EB titer

Following the results of our transwell experiments, we used phenotypically non-piliated (P-) *N. gonorrhoeae* or hyperpiliated (Δ*pilT*) *N. gonorrhoeae* in the co-infection experiment. Type 4 pili (T4P) are retractable and the PilT ATPase is responsible for retraction of the pilus. In our experiment, we utilized a gonococcal mutant MS11Δ*pilT*::Kan^R, which is constitutively hyperpiliated due to its inability to retract its pili. We hypothesized that we would see a moderate decrease in EB titer with non-piliated and Δ*pilT N. gonorrhoeae,* but not as great as observed with piliated *N. gonorrhoeae*. Figure 4D shows that there were slight, but not statistically significant differences between the quantity of cell-associated *N. gonorrhoeae* after the co-infection, and the *C. trachomatis* genome copy per well did not change significantly between the co-infection conditions (Fig. 4E). However, we observed a chlamydial EB titer decrease of just under one log when non-piliated or hyperpiliated *N. gonorrhoeae* were grown with *C. trachomatis*. This supported the observation from the transwell experiment that medium modification by *N. gonorrhoeae* was sufficient to alter the *C. trachomatis* EB production and while T4P exacerbates the persistence-like response, they are not solely responsible for it.

Inhibition phenotype is not due to tryptophan starvation

Given that the previous observations suggested that *C. trachomatis* was entering an aberrant state during co-infection with *N. gonorrhoeae*, we decided to investigate whether the phenotype could be rescued by tryptophan supplementation. One

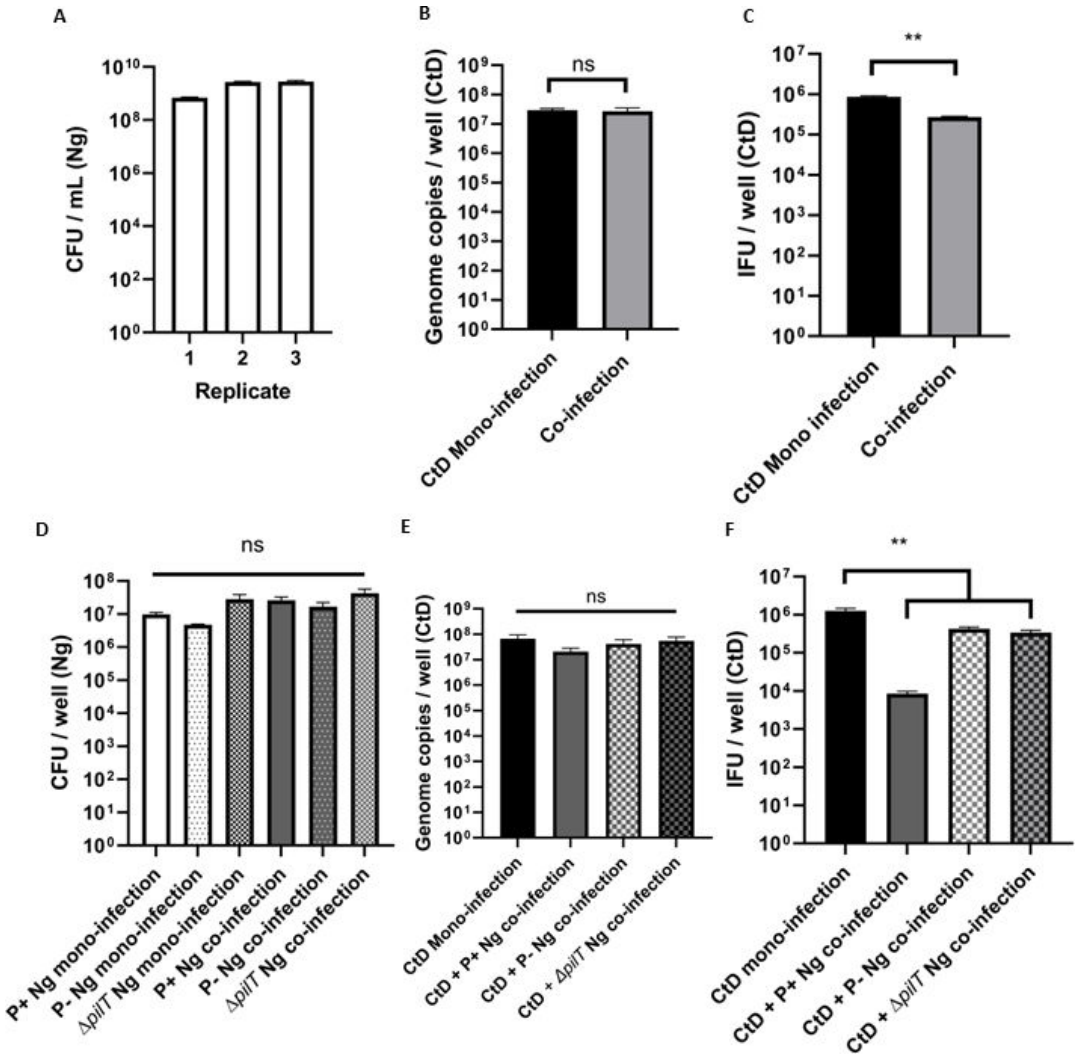

FIG 4 Aberrant Chlamydial phenotype is exacerbated by type 4 pili (T4P) but still observed during non-contact co-culture. HeLa cells were pre-infected with *C. trachomatis* D (CtD) and then challenged with *N. gonorrhoeae* (Ng) grown in the upper reservoir of 0.4 µm transwells for 12 hours. (A) *N. gonorrhoeae* grew to a stationary phase (10^8 CFU/mL) in all three biological replicates. (B) Genome copy number for *C. trachomatis* D remained equivalent between mono- and co-infected conditions. (C) Fewer IFU/well were produced during *C. trachomatis–N. gonorrhoeae* co-culture compared with a *C. trachomatis* mono-infection. Each experiment was performed in technical triplicate and three biological replicates were performed on separate days. IFU/well titers are significantly different by Student's *t*-test, $^{**}P < .01$. HeLa cells were pre-infected with *C. trachomatis* D (CtD) then challenged with piliated (P+), non-piliated (P-), or hyperpiliated (Δ*pilT*) *N. gonorrhoeae* (Ng) for 12 hours. (D) Co-infection did not significantly increase gonococcal attachment to monolayers for any of the piliated variants. (E) Chlamydial genome copy did not significantly change between mono-infection and any of the co-infection conditions. (F) Co-infections using non-piliated or hyperpiliated *N. gonorrhoeae* produced a decrease in Chlamydial EB titer. Error bars are the standard error of the mean (SEM) and ** represents $P < 0.01$ (Tukey's multiple comparisons test). Results are significantly different by one-way ANOVA ($P < 0.01$).

mechanism of Chlamydial persistence induction is tryptophan starvation (32). *C. trachomatis* is a tryptophan auxotroph and serovars D – L3 have only the genes for the final steps of the tryptophan biosynthetic pathway: *trpAB* which encodes the two subunits of the tryptophan synthase enzyme and permits synthesis of tryptophan from its precursor indole. *C. trachomatis* cannot synthesize indole but can scavenge it in the genital tract where it is produced by *Prevotella* spp (33). We hypothesized that gonococcal growth could potentially deplete tryptophan to a concentration too low to permit Chlamydial growth. To test this, we repeated the co-infection and supplemented the medium with 400 µM tryptophan or 10 µM indole in addition to NEAA and $Fe(NO_3)_3$. Previous research has shown that these concentrations are sufficient to rescue *C. trachomatis* from IFN-γ-induced persistence (33). However, co-infections supplemented

with indole or tryptophan did not restore Chlamydial EB development and the EB titer remained equivalent to that of an un-supplemented co-infection while Chlamydial genome copy number and adherent gonococcal CFU/well remained equivalent (Fig. 5A through C).

LGV biovar *C. trachomatis* is not sensitive to the effects of *N. gonorrhoeae* co-infection

We were curious whether this reduced EB titer effect was limited to a urogenital biovar of *C. trachomatis*. We repeated the basic co-infection experiment using an LGV serovar *C. trachomatis* serovar L2. L2 is reportedly more virulent than serovar D, so to account for this increased infectivity, we lowered the infection inoculum MOI from 5 to 1 for this experiment (34). All other parameters of the experiment remained unchanged. As with previous co-infections, the quantity of attached *N. gonorrhoeae* and the GC number for serovar L2 did not change between co- and mono-infections (Fig. 6A and B). We were surprised to find that serovar L2 EB titer did not change when *C. trachomatis* was challenged with *N. gonorrhoeae* (Fig. 6C). We examined the CtL2 inclusions using SIM and did not see aberrant bodies during the co-infection (Fig. 6E). These findings suggest that the *C. trachomatis* LGV serovar is resistant to the effect of *N. gonorrhoeae* during co-infection. As the genomes for both serovars D and L2 have been sequenced, this enables us to compare the two strains and hypothesize mechanisms for this observation. We tentatively explored the putative Chlamydial cytotoxin by repeating the co-infection with the mouse strain, *C. muridarum*. We hypothesized that whether serovar D's putative partial cytotoxin was responsible for the inhibition phenotype, we would observe an increased effect with *C. muridarum*, which has a complete cytotoxin coding sequence (CDS) and has been previously demonstrated to exacerbate

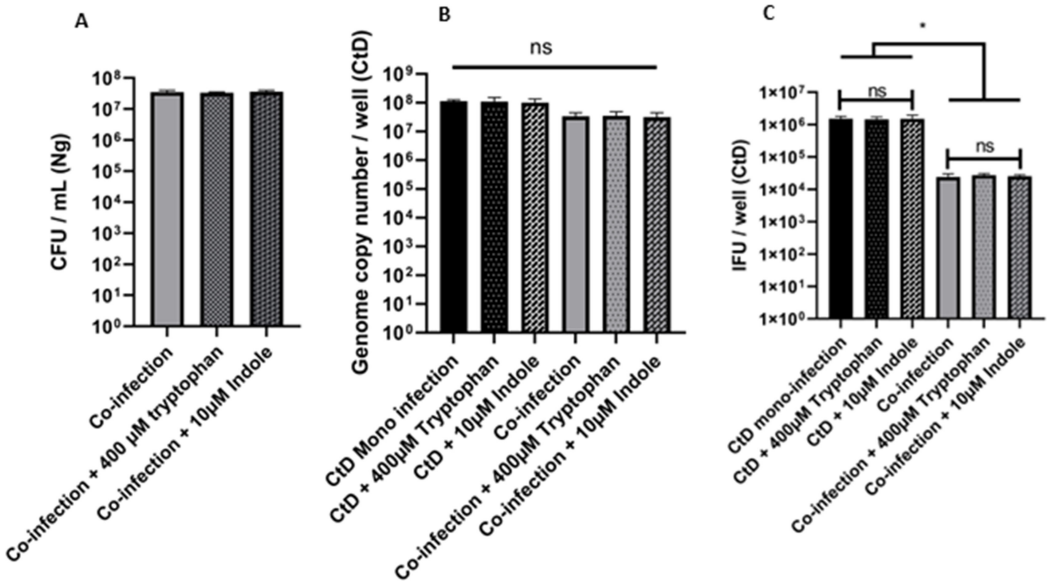

FIG 5 Supplementation of co-infection with 10 µM indole or 400 µM tryptophan does not restore the elementary body (EB) titer to mono-infection levels. HeLa cells were pre-infected with *C. trachomatis* CtD and then challenged with *N. gonorrhoeae* (Ng) with or without tryptophan or indole for 12 hours of growth. (A) *N. gonorrhoeae* (Ng) CFU/mL after 12 hours of co-infection with C. trachomatis D ± tryptophan or indole. (B) Quantitative PCR measurement of genome copy number for all forms of *C. trachomatis* (CtD) (EBs, RBs, and ABs) after 24 hours of growth in HeLa cells including 12 hours of co-infection with or without *N. gonorrhoeae* ± tryptophan or indole. (C) Quantitative immunofluorescence analysis of *C. trachomatis* D (CtD) EB titer after 24 hours of growth in HeLa cells including 12 hours of co-infection with or without *N. gonorrhoeae* ± tryptophan or indole. Each experiment was performed with three technical replicates and each experiment was performed three times. Error bars are the standard error of the mean (SEM) and * represents $P < 0.05$ (Tukey's multiple comparisons test). Results are significantly different by one-way ANOVA ($P < 0.01$).

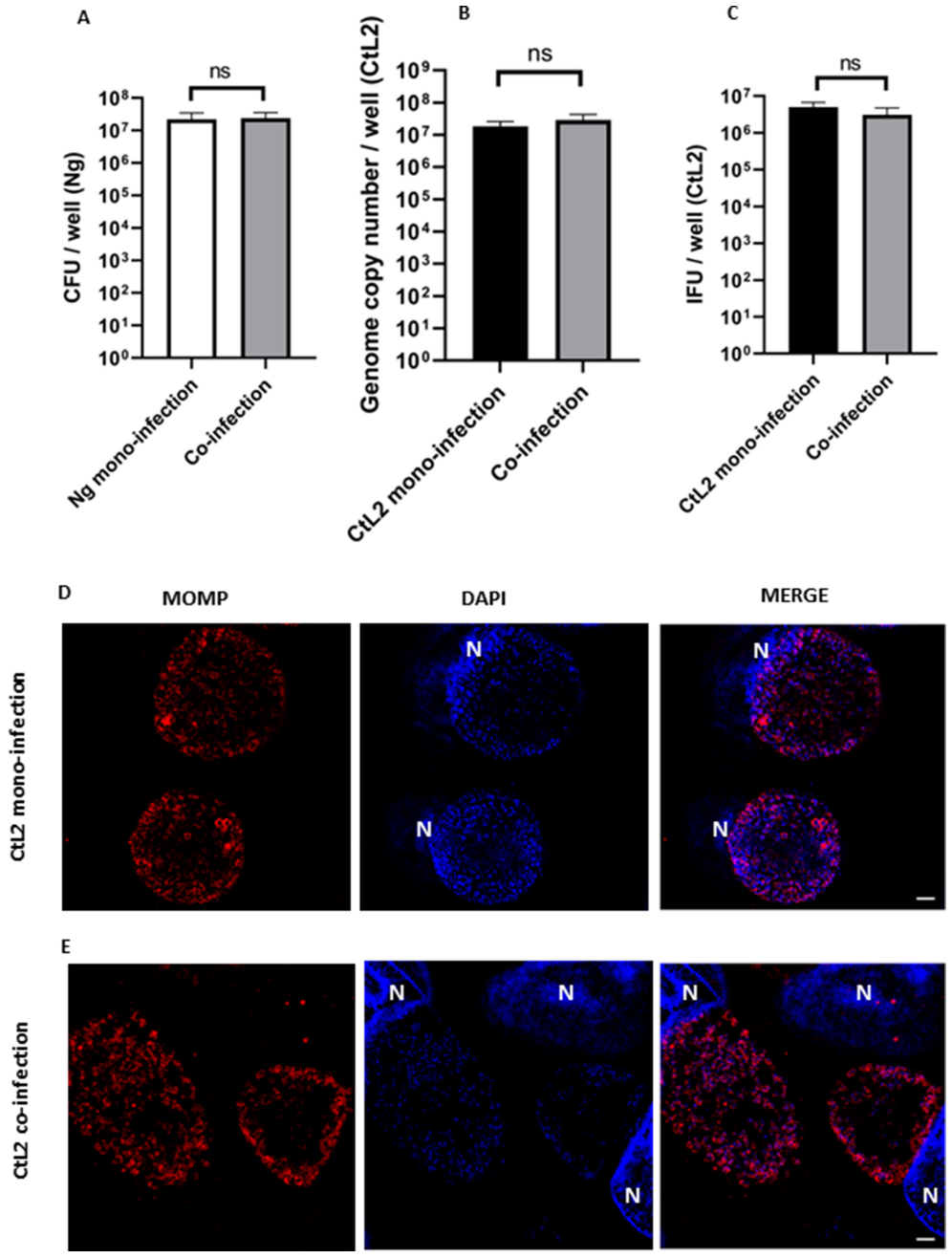

FIG 6 *C. trachomatis* serovar L2 is resistant to the EB titer reduction during co-infection with *N. gonorrhoeae*. *C. trachomatis* (CtL2) pre-infected HeLa cells were challenged with live *N. gonorrhoeae* (Ng) for 12 hours of growth. (A) *N. gonorrhoeae* (Ng) CFU/mL after 12 hours of co-infection with or without *C. trachomatis* L2. (B) Quantitative PCR measurement of genome copy number for all forms of *C. trachomatis* (CtL2) (EBs, RBs, and ABs) after 24 hours of growth in HeLa cells including 12 hours of co-infection with or without *N. gonorrhoeae*. (C) Quantitative immunofluorescence analysis of *C. trachomatis* L2 (CtL2) EB titer after 24 hours of growth in HeLa cells including 12 hours of co-infection with or without *N. gonorrhoeae*. Statistical analyses by Student's *t*-test. Error bars are SEM. All experiments were performed in triplicate and repeated a total of three times. There is no significant difference between the mono- and co-infections when measured by Student's *t*-test. Super illumination microscopy (SIM) fluorescence microscopy representative images of (D) CtL2 mono-infection and (E) CtL2 - *N. gonorrhoeae* co-infection. Chlamydial cells are the same size as mono-infected cells after 12 hours of co-infection. Cells were fixed and permeabilized with methanol and 0.5% Triton X-100. *Chlamydia* appears red and DNA appears blue. Host cell nuclei are noted with "N." White scale bars are 2 µm.

cytotoxin-dependent phenotypes (31). By contrast, the EB titer in *N. gonorrhoeae–C. muridarum* co-infection was only reduced by twofold (Fig. S2).

Co-infection with *N. gonorrhoeae* alters *C. trachomatis* gene expression

Several studies into Chlamydial persistence in epithelial cells have attempted to identify a profile of genes that are upregulated or downregulated because of persistence (34–37). However, there is no clear single transcriptome pattern associated with all the methods used to induce persistence.

We selected four genes to probe and normalized the transcripts to chlamydial 16S. All the primers used for these genes are listed in Table S1. We chose to probe *hctB* as this is a late gene associated with the transition from RB to EB; *ftsK* as this gene has previously been observed unchanged in some transcript studies but upregulated in others (35–37). Lastly, we chose *trpB* and *recA* as these are two genes upregulated in IFN-γ-induced persistence. Table 1 shows the log2 fold change (log2 FC) for both *C. trachomatis* serovar D and *C. trachomatis* serovar L2 co-infections with *N. gonorrhoeae* when compared to mono-infections, and how these results compare to four transcript analyses in the literature. *C. trachomatis* serovar L2 showed no significant differences between mono- and co-infections. *C. trachomatis* serovar D showed a significant 4.6 log2 FC decrease in *hctB* transcripts. The other genes did not show significant differences between mono- and co-infection.

DISCUSSION

The findings from this study have revealed an outcome of *C. trachomatis–N. gonorrhoeae* co-infection that we did not anticipate but is fascinating, nonetheless. There is no apparent cooperation between these two bacterial pathogens, at least when investigated in the narrow context of epithelial cell co-infection. Instead, we found that when an existing mid-cycle *Chlamydia* infection (12 hpi) is challenged by extracellular *N. gonorrhoeae* brought into contact with the host cell membrane, *C. trachomatis* continues growing and dividing but does not transition to the infectious EB state as readily as they do in a mono-infection. The Chlamydial response is not immediate, otherwise we would see a lack of EB development as EBs are not detectable until 18 hours in the developmental cycle. While this is not an apparent benefit for *Chlamydia*, an innate ability to delay a complete developmental cycle culminating with the release of progeny bacteria, until an environment becomes less hostile can be interpreted as a "benefit." Persistence has been observed in *C. trachomatis* experiments for decades and shown to be induced by a variety of stimuli (12). These stimuli include but are not limited to nutrient deprivation, antibiotic use, and iron deprivation. As *N. gonorrhoeae* is known to be highly inflammatory and recruit large numbers of immune cells to the site of infection, this would present a challenge for EBs upon cell lysis as increased risk of phagocytosis by neutrophils would reduce the likelihood of re-infection into a new cell. *C. trachomatis* serovar D EBs can infect neutrophils but this is an abortive infection as the transition from EB to RB leads to cytotoxic death before the developmental cycle can be completed (38). As such, entering a viable but non-replicative state would further

TABLE 1 Late gene *hctB* is downregulated during *C. trachomatis* serovar D co-infection[a]

Gene	CtD FC	*P* value	CtL2 FC	*P* value	IFN (37)	HSV (35)
HctB	−4.6489	0.0285	−0.7593	0.1398	Down	N/A
FtsK	−0.9555	0.2788	0.3418	0.7236	No change	No change
TrpB	−1.8177	0.1442	−2.0526	0.3107	Up	N/A
RecA	−0.2669	0.7850	0.3114	0.7564	Up	N/A

[a]cDNA was prepared from RNA from *C. trachomatis* (CtD or CtL2) and *N. gonorrhoeae* co-infected HeLa cells. Real-time quantitative PCR was performed for *C. trachomatis* genes *hctB, ftsK, trpB,* and 16S. Transcripts were normalized to 16S for each experiment. FC equals log2 fold change for each averaged transcript. The IFN column shows the transcript change measured during IFN-induced persistence, and the HSV column shows the transcript change measured during herpes simplex virus (HSV)–*C. trachomatis* co-infection. Each experiment was performed three times with three technical replicates. Statistical analysis was performed by Student's *t*-test and $P \leq 0.05$ is considered significant.

prolong the longevity of *C. trachomatis* within epithelial cells in the genital tract until the *N. gonorrhoeae* infection had been cleared.

When we added gonococci treated in a manner where they were dead (isopropanol) or unable to grow (gentamycin), we did not see a decrease in Chlamydial EB titer. We hypothesized that our previous observation could be due to receptor engagement between gonococci and the HeLa cell resulting in a detrimental cell signaling event causing stress on the developing *Chlamydia* which was sufficient to produce persistence. *N. gonorrhoeae* possesses several pathogenesis factors used for colonization and survival in the human host (39). Outer membrane structures include porin, T4P, opacity-associated proteins (Opa), and lipooligosaccharide (LOS). Porin (PorB) is a voltage-gated pore involved in small nutrient acquisition and ion exchange (40). PorB is capable of translocation into mammalian cell membranes *via* outer membrane vesicles (OMV) causing calcium influx leading to apoptosis (41, 42). PorB has also been implicated in the gonococcal invasion of epithelial cells (43, 44) and contributes to serum resistance through complement component binding (45).

There are 11 Opa proteins in *N. gonorrhoeae* and, in theory, any combination from all or none of them can be expressed by the bacteria (46). These proteins bind to cellular carcinoembryonic antigen-related cell adhesion molecules (CEACAMs), some of which are found on epithelial cells and are involved in the mucosal immune response (47). However, in our study, we used Opa- bacteria to prevent confounding results which could be Opa dependent. Therefore, we did not consider Opa involvement in the *C. trachomatis* response to co-infection.

LOS in *N. gonorrhoeae* is present in the outer leaflet of the outer membrane and is similar to lipopolysaccharide (LPS; endotoxin) in other Gram-negative bacteria but lacking the O-antigen polymer (48). Like the other outer membrane structures discussed above, it is important for adherence, invasion, and immune system evasion (49–51). Lipid A is a danger signal to the immune system and is recognized by toll-like receptor 4 (TLR4), which leads to activation of inflammatory transcription factors and chemokine and cytokine release (52).

T4P are dynamic structures that have been previously demonstrated to exert tremendous force on the eucaryotic plasma cell membrane. When T4P retracts, they produce forces of 80–100 pN on HeLa cells which leads to enhanced expression of infection-regulated genes, most of which are under the control of mitogen-activated protein kinases (MAPKs) (16, 18). During intracellular growth, the Chlamydial inclusion is encased in actin, intermediate filaments, and microtubules which stabilize the inclusion and aid in the transport of essential nutrients (53, 54). Loss of this stability could result in the enlargement of the inclusion. It is difficult to hypothesize if this could be the case during *C. trachomatis–N. gonorrhoeae* co-infection as Howie et al. showed that when magnetic beads are used to exert forces on eucaryotic cells equivalent to that of pilus retraction, actin recruitment is increased not decreased (16). Actin is recruited toward the magnetic beads; therefore, it could theoretically be recruited away from the inclusion during co-infection.

Our gentamycin assay showed that when 12 hours of gonococcal conditioned medium is removed and replaced with fresh medium with or without gentamycin, the *Chlamydial* EB titer recovers to levels similar to a mono-infection. As gentamycin is a plasma membrane impermeable antibiotic, we did not expect the gentamycin to have a strong detrimental effect on EB titer. However, as shown in Fig. 3, we did not expect the gentamycin-free medium replacement replicates to have a *C. trachomatis* EB titer recovery not significantly different from the gentamycin-containing wells. We hypothesize that this is due to an unidentified factor in the *N. gonorrhoeae* conditioned medium which inhibits *C. trachomatis* development. In addition, most *N. gonorrhoeae* in the co-infection are non-adherent and are removed with the medium. This creates a window of opportunity for *C. trachomatis* to transition back to an actively growing state before the gonococci can multiply to high CFU/mL again. Further experiments are required to identify the factor within the medium causing this phenotype. *N. gonorrhoeae* secretes

a variety of molecules that contribute to virulence including peptidoglycan fragments, LOS, DNA, and OMV all of which are pathogen-associated molecular patterns recognized by HeLa cells.

The finding that *C. trachomatis* serovar L2 is resistant to the inhibitory effect generated by co-infection is especially interesting. A recent study on *C. trachomatis–N. gonorrhoeae* co-infection also saw decreased *C. trachomatis* EB titer and inclusion size (27). In this study, HeLa cells were incubated with *N. gonorrhoeae* for 24 hours before being infected with *C. trachomatis* and cycloheximide was added throughout the experiment. Cycloheximide prevents the translation of new proteins in eucaryotic cells, meaning that the host cells are restricted from responding to *Chlamydial* triggers except with proteins that are already present. A different urogenital *C. trachomatis* serovar (serovar E) and strain of *N. gonorrhoeae* (FA1090) were also used in this study. The authors also noted that they saw a similar effect with *C. muridarum*. In our study, we saw a similar but diminished effect with *C. muridarum*. When comparing the genomes of the two urogenital serovars (D and E), *C. trachomatis* serovar L2, and *C. muridarum*, one notable observation is the presence of genes in the Plasticity Zone (PZ). Of the pathogenic *Chlamydia*, *C. muridarum* has the largest PZ with several large CDS for putative Chlamydial cytotoxins. Serovar L2 does not have any CDS which resemble the putative cytotoxin genes, but Serovar D and E both have genes with some cytotoxin gene homology. However, when we used *C. muridarum* in co-infection with *N. gonorrhoeae,* we did not see a 100-fold decrease in EB titer as we observed with serovar D. This suggests that the *Chlamydial* cytotoxin is not playing a role in the phenotype we have observed.

When comparing *C. trachomatis* serovars D (urogenital) and L2 (LGV), an important difference is the site of infection. A typical urogenital infection may ascend to the upper reproductive tract (in women), but LGV strains invade from the genital tract into the regional lymph nodes. An infected individual first develops a small painless ulcer or papule which may resolve or progress to a secondary stage with lymphadenopathy. If untreated, the disease can result in necrosis and fibrosis. Disseminated gonorrhea leading to complications such as endocarditis, dermatitis, or arthritis results from transit *via* the bloodstream. In a clinical situation, an LGV *C. trachomatis* serovar is unlikely to encounter *N. gonorrhoeae* as it does not invade lymph nodes. The LGV serovars may have undergone rounds of natural selection and lost the ability to respond to gonococcal threat as it was simply not needed. To probe this hypothesis further, the co-infection model would need to be repeated with other LGV serovars: L1, L2b, and L3. Repeating co-infections with other serovars in the urogenital and LGV biovars would start to disentangle the potential avenues for exploration. For example, LGV strains L1, L2, L2b, and L3 are still genetically different and if the phenotype observed here with serovar L2 is not reproducible with L1, L2b, and/or L3, that would tell us that the observation is not as binary as LGV vs urogenital biovar specificity.[551313,5614] There are many future directions arising from these initial observations. These can be broken down into three distinct questions: (1) What is *C. trachomatis* modulating in its developmental cycle to generate this phenotype? (2) What is the gonococcus doing to produce the *C. trachomatis* persistence-like phenotype? And (3) What is the role of the host cell in this phenotype? A future direction we have already undertaken is a dual RNA-seq analysis of the *C. trachomatis* and *N. gonorrhoeae* transcriptomes during *in vitro* co-infection (data in analysis). We observed extensive transcriptome changes in *C. trachomatis* serovar D with generally more downregulation than upregulation of genes. When considering the role of the gonococcus, there are many secreted virulence factors produced by *N. gonorrhoeae* which would accumulate in the shared culture medium These include peptidoglycan fragments, OMV and DNA.

In this study, we limited *N. gonorrhoeae* to extracellular growth only by using phenotypically Opa- bacteria. Clinical isolates typically express some combination of Opas meaning they are potentially invasive to epithelial cells. With that in mind, it would be important to investigate how cellular invasion by *N. gonorrhoeae* affects *C. trachomatis* inclusion development.

Lastly, the question of the effect on the host cell is an avenue we did not explore. Previous transcriptome analysis of *C. trachomatis* infection of HeLa cells has revealed that the number of host cell transcripts is reduced 10-fold (from approximately 100 million reads to approximately 10 million) at 24 hpi (55). In the same study, Humphrys et al. identified that upregulated eucaryotic transcripts included pro-fibrotic genes as well as "immune dampening" genes for antimicrobial peptides and extracellular matrix mucin expression. Overall, we have identified a distinct phenotype for urogenital *C. trachomatis* during co-habitation with *N. gonorrhoeae* which may translate over to clinical infections. This phenotype is defined by decreased EB production, but an absence of an effect upon *N. gonorrhoeae*. It refutes our initial hypothesis of a symbiotic effect on these two pathogens during co-infection. We developed a reliable *in vitro* model using immortalized cervical epithelial cells and have shown that the impact of co-infection on extracellular *N. gonorrhoeae* is negligible. Previous co-infection studies have focused on the observations of chlamydial development when epithelial cells are pre-infected with *N. gonorrhoeae*, or both *C. trachomatis* and *N. gonorrhoeae* at the same time (27, 56). Therefore, this work contributes to the fledgling field of *C. trachomatis–N. gonorrhoeae in vitro* co-infection by revealing how a productive mid-cycle chlamydial infection responds to the new challenge of *N. gonorrhoeae* entering its environmental niche.

MATERIALS AND METHODS

Bacterial strains

C. trachomatis serovar D/UW-3/CX (ACE022) and *C. trachomatis* serovar L2434/Bu (ACE004) were originally provided by Catherine O' Connell (University of North Carolina, Chapel Hill, NC) and Harlan Caldwell (National Institutes of Health, Bethesda, MD), respectively. *C. muridarum* Nigg M9 (ACE132) was originally provided by Roger Rank (University of Arkansas for Medical Sciences, Little Rock, AR). Each serovar was expanded and stored as multiple single-use aliquots. The inclusion forming unit (IFU) per mL was calculated using an immunofluorescence assay (57) for each serovar batch. *N. gonorrhoeae* strain MS11 was provided by Joseph Dillard (University of Wisconsin, Madison, WI) and freshly streaked from a frozen stock onto gonococcal medium base agar (GCB) with Kellogg's supplements (58). Phenotypically piliated (P+) and Opa-negative (Opa-) colonies were visualized using a stereo microscope (Leica), and approximately 10 colonies were passaged to a fresh GCB plate and allowed to grow for 12 to 13 hours (h). This bacterial lawn was swabbed using Dacron swabs (Puritan) and resuspended in 1× phosphate-buffered saline (PBS) to prepare inocula for experiments. P- MS11 *N. gonorrhoeae* was isolated by colony morphology and passaged to give a P-stock. Hyperpiliated MS11 was prepared by transforming MS11 with gDNA extracted from FA1090 "Opaless" Δ*pilT*:KanR *N. gonorrhoeae* provided by Dr. Alison Criss (University of Virginia). Kanamycin-resistant colonies were passaged and confirmed by PCR using primers pilT F and pilT R, also provided by Dr. Alison Criss. Phenotypically, these clones were visibly hyperpiliated. MS11 Δ*pilT*:KanR was stocked and used in subsequent experiments.

Eucaryotic cell lines

HeLa-USU, a derivative of HeLa-CCL2 endocervical epithelial cells originally sourced from the American Type Culture Collection but passaged at the Uniformed Services University (USU) (59), was used in all co-infection and other tissue culture assays. The HeLa cells were maintained in Roswell Park Memorial Institute medium (RPMI 1640, Gibco) supplemented with heat-inactivated 10% fetal bovine serum (FBS, Gibco). When used in IFU titer assays, HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FBS. Cells expanded from a single frozen HeLa aliquot were not passaged more than 30 times and were tested for mycoplasma contamination at passages 1, 12, and at the end of use. *C. trachomatis* L2 434/Bu was originally

expanded in L2 mouse fibroblast cells before the start of this study. L2 cells were routinely grown in DMEM supplemented with 10% FBS.

C. trachomatis–N. gonorrhoeae co-infection

Two microliters of 6.2×10^4 cells/mL HeLa cells was plated per well in a 12-well plate (Corning) 24 hours before infection with *C. trachomatis* serovar D. Estimating that confluency is approximately 5×10^5 cells/well, and with the assumption that HeLa cells divide every 24 hours, this approach considers the physical requirements of the growing eucaryotic cells throughout the co-infection experiment and minimizes the impact of overcrowding on the experiment outcome. Twenty-four hours after seeding the HeLa cells, the medium was removed, monolayers were washed once with PBS, and the medium was replaced with 1.5 mL RPMI plus 10% FBS. *C. trachomatis* serovar D was diluted to 1.2×10^7 IFU/mL in sucrose phosphate glutamic acid buffer (SPG), and 100 µL added to each well, that is, a MOI ≈ 5. HeLa cells that would receive *N. gonorrhoeae* only (see below) were mock infected with 1.5 mL RPMI plus 10% FBS and 100 µL SPG. Cells plus bacteria were rocked for 30 minutes (min) at 37°C in 5% CO_2, then centrifuged at 500× *g* at room temperature for another 30 minutes. Media and inocula were removed by aspiration and monolayers were washed twice with PBS. One microliter of RPMI plus 10% FBS was added to each well and the infection was allowed to progress for 12 hours. At the start of the co-infection, *N. gonorrhoeae* were swabbed from a 12- to 13-hour-old lawn into 3–5 mL of PBS and vigorously vortexed to resuspend the bacteria. Bacterial CFU/mL was estimated by measuring the optical density (OD) at wavelength 550 nanometers (OD_{550}) using a Biomate S3 spectrophotometer (Thermofisher) and using the standard equivalent of 0.2 OD_{550} ≈1×10^8 CFU/mL. Bacteria were diluted to approximately 1.7×10^6 CFU/mL in RPMI plus 10% FBS. The *C. trachomatis*-infected monolayers were washed twice with PBS, and 1.5 mL (2.5×10^6 CFU, MOI ≈ 10) *N. gonorrhoeae* was added to each well. *C. trachomatis* only wells received 1.5 mL RPMI. To facilitate *N. gonorrhoeae* attachment to the HeLa cells, the plates were centrifuged at 600× *g* for 4 minutes at room temperature. Immediately after centrifugation, the inoculum was removed and each well was washed twice with PBS. Cell monolayers were incubated in 1 mL of supplemented medium [RPMI plus 10% FBS, plus non-essential amino acids (NEAA) and 12 µM iron (III) nitrate ($Fe(NO_3)_3$] at 37°C in 5% CO_2 for 12 hours. To harvest bacteria from the wells at 12 hpci, the media was removed, and monolayers were gently washed with PBS twice. HeLa cells were detached and lysed by vigorously rolling five-5 mm glass beads (Fisher Scientific) across each monolayer. Lysate was collected in 1 mL of PBS, and the well surface was washed with 1 mL of PBS twice. The wash was added to the lysate to bring the sample total to 3 mL. The samples were vortexed well, and 100 µL was stored in SPG at −80°C for Chlamydial EB enumeration. One microliter was collected separately for Chlamydial genome copy (GC) number calculation. Finally, 100 µL was serially diluted 1:3 in gonococcal medium base liquid (GCBL) for *N. gonorrhoeae* enumeration (see below). Figure 1A shows the timeline for the co-infection. When *C. trachomatis* serovar L2 was used in the co-infection, the MOI was reduced to 1.

When *C. muridarum* was used in the co-infection, the incubation periods were shortened to account for its relatively faster developmental cycle. *C. muridarum* was infected at an MOI of 1 onto sub-confluent HeLa cells as described above. Infected cells were incubated at 37°C in 5% CO_2 for 8 hours. The medium was removed and *N. gonorrhoeae* was infected by centrifugation at an MOI of 10 as described above. The co-infection was incubated for an additional 10 hours and then harvested as described above. We performed a growth curve using *C. muridarum* and identified 18 hpi as a feasible timepoint because EBs were above the limit of detection (data not shown).

Colony counts for *N. gonorrhoeae* titer

One hundred microliters of co-infection harvest sample was serially diluted 1:3 (100 µL sample plus 200 µL GCBL) and 3–10 µL spots were added onto pre-dried GCB agar plates. Plates were incubated at 37°C with 5% CO_2 overnight. Triplicate spots with 20–200 colonies were counted using a stereomicroscope and used to calculate CFU/well. Samples with <20 colonies counted per spot at 10^0 were considered below the limit of detection.

Immunofluorescence for EB titer

HeLa cells were pre-seeded onto acid-washed coverslips in 24-well plates (Corning) at 1.3–1.5 × 10^5 cells/well in DMEM plus 10% FBS. One or two days later, media were removed from the monolayers and 1:10 serial dilutions of the previously harvested EBs were prepared in SPG. The medium was removed from the 24-well plates and replaced with 100 µL of diluted sample. Plates were gently rocked at 37°C with 5% CO_2 for 2 hours to facilitate the adsorption of bacteria to the HeLa cell membrane. Inoculum was removed and replaced with 1 mL DMEM plus 10% FBS supplemented with 1 µg/mL cycloheximide. When this method was used to count EBs produced during co-infection experiments, 20 µg/mL gentamycin was included to prevent *N. gonorrhoeae* growth. Plates were incubated at 37°C in 5% CO_2 for 42–48 hpi. Cells were fixed and permeabilized by removing the growth medium and adding ice-cold 100% methanol for 20–30 minutes. Methanol was promptly removed, and cells were washed in IFA wash buffer (1× PBS, 0.1% bovine serum albumin, 0.05% Tween-20). *C. trachomatis* inclusions were stained using Pathfinder anti-*Chlamydial* LOS antibody (BioRad, 30701) for 30–60 minutes. Coverslips were individually washed by dipping 10–20 times in 50–100 mL of IFA wash buffer, blotting on a paper towel, and mounting on glass slides with Fluoro-mount-G (Southern Biotech). Stained inclusions across duplicate coverslips were counted at 200× magnification using an inverted epifluorescence microscope (Zeiss). The limit of detection is considered 20 inclusions/coverslip, and, wherever possible, coverslips with ≥20 inclusions were used for EB titer calculation. Data are presented as average IFU/well ± standard error of the mean (SEM).

DNA extraction by phenol-chloroform

One microliter of co-infection harvest sample was centrifuged at 20,000 *g* for 5 minutes at room temperature and then the pellet was resuspended in 400 µL Lysis buffer (Tris-EDTA pH 8.0, 1% sodium dodecyl sulfate) and incubated at room temperature overnight. Total DNA was extracted using a standard phenol:chloroform protocol (60). Briefly, 200 µL of phenol and then 200 µL chloroform were added to each sample and vortexed well. Samples were centrifuged at 16,000 *g* for 5 minutes to separate phases and 300 µL of the aqueous phase transferred to a new microfuge tube. An equal volume of chloroform was added and the sample vortexed and centrifuged at 16,000 *g* for 5 minutes. Two hundred microliters of the aqueous phase was transferred to a new microfuge tube and 20 µL 3M sodium acetate pH 5.2 and 400 µL of ice-cold 100% ethanol was added to precipitate DNA. Samples were vortexed and incubated at −20°C for 1 hours overnight. DNA was pelleted by centrifuging at 19,000× *g* in a microfuge at 4°C for 30 minutes. The supernatant was carefully removed and replaced with ice-cold 70% ethanol. Samples were centrifuged at maximum speed at 4°C for 15 minutes and the supernatant was carefully removed. DNA pellets were allowed to air dry, and dissolved in 50 µL molecular biology grade water, and the DNA concentration was measured on a Nanodrop 2000 spectrophotometer (Fisher Scientific). Samples were stored at −20°C until used in a quantitative polymerase chain reaction (qPCR).

qPCR for *C. trachomatis* genome copy number

The *C. trachomatis* 16S rRNA gene was amplified *via* qPCR using primers: u_16 s F TAGTG TGTGAGGGGATAAATTGAGAG and u_16s R GTTTAGCATCTATACTGGCCTGCATTCT (61). The *C. muridarum* 16S rRNA gene was amplified *via* qPCR using primers Cm 16S F CGCCTGA GGAGTACACTCGCAAGG and Cm 16S R CCAACACCTCACGGCACGAG (62). For each qPCR run, a fresh standard curve was prepared to compare measured threshold values (Cq) from a known concentration of *C. trachomatis* or *C. muridarum* genomic DNA (gDNA.). A 1:10 dilution series standard curve from 4×10^2 to 4×10^8 genome copies was amplified in triplicate for each qPCR run. A water template negative control was included each time. Before routine use in quantification assays, both an absence of amplification of HeLa or *N. gonorrhoeae* gDNA and good amplification efficiency were confirmed for these primer pairs. Each technical replicate was amplified once in a final volume of 10 µL using Luna Universal qPCR Mastermix (Qiagen), 0.25 µM of each primer, and with cycling conditions: 95°C/1 minute [95°C/15 s, 60°C/30 s, plate read] × 45 cycles. A melt curve was also performed after the amplification to ensure a single target had been amplified.

Giemsa staining and microscopy

Co-infections were performed as described above, using the same MOIs but in 24-well plates with covered glass bottoms (Nunc). Medium was removed from monolayers, and the cells were fixed at room temperature using 4% paraformaldehyde for 10 minutes. Cells were washed with 1× PBS and stained with a freshly prepared 1:40 dilution of Giemsa stock solution (7.6 mg/mL) for 2 hours at room temperature. Excess dye was removed and replaced with 1× PBS for imaging. Cells were viewed at 1,000× with oil immersion using the brightfield setting on an inverted epifluorescence microscope (Zeiss) and imaged with Zen Blue software (Zeiss).

Fluorescence microscopy and imaging

Co-infections were performed as described above, using the same MOIs. HeLa cells were cultured on acid-washed coverslips and after the co-infection, the monolayer was gently washed twice with PBS and then fixed with room temperature methanol for 5 minutes. Monolayers were washed with PBS three times and then incubated with 0.5% Triton-X100 for 5 minutes. Monolayers were washed with PBS and then blocked with 3% BSA for 1 hour. Coverslips were incubated with α-MOMP primary antibody (Lifespan Biosciences) for 1 hour and then washed with 3% BSA. Coverslips were incubated with a secondary antibody conjugated to Alexfluor 594 (Invitrogen) for 1 hour and then washed with 3% BSA. Coverslips were mounted on slides with Fluoromount-G and sealed with nail polish. Fluorescent images were obtained using super-resolution microscopy *via* a Zeiss ELYRA PS.1 set to SIM-imaging mode.

Gentamycin recovery assays

The co-infection protocol as described above was performed, but at 12 hpci, the media were removed from all wells, the monolayers were washed twice with 1X PBS, and fresh medium supplemented with 10% FBS, 1× NEAA, and 12 µM Fe^{3+} with or without 20 µg/mL gentamycin was added to the wells. The co-infection continued for an additional 12 hours at 37°C, 5% CO_2. Figure 3 shows the timeline for this modification of the co-infection procedure. At 24 hpci, the media were removed, and the monolayers were washed twice with 1× PBS. HeLa cells were detached and lysed by vigorously rolling five- 5 mm glass beads across each monolayer. The lysate was collected in 1 mL of PBS and the well surface was washed twice with 1 mL of PBS. The wash was added to the collected lysate to bring the sample total to 3 mL. The samples were vortexed well and 100 µL was stored in SPG at −80°C for Chlamydial EB enumeration. One microliter was collected separately for Chlamydial GC number enumeration. Finally, 100 µL was serially diluted 1:3 in GCBL for *N. gonorrhoeae* enumeration as described above.

Isopropanol and gentamycin killing assays

The co-infection was performed as described above. Before inoculating the wells with *N. gonorrhoeae*, 10^8 bacteria were centrifuged at 10,000× *g* for 3 minutes and resuspended in 1 mL 70% isopropanol for 10 minutes at room temperature, then centrifuged again and resuspended in supplemented medium. This inoculum was further diluted in the medium and added to the appropriate wells for infection. At the same time, another 10^8 *N. gonorrhoeae* were centrifuged at 10,000× *g* for 3 minutes and resuspended in 1 mL supplemented medium with 20 µg/mL gentamycin. This inoculum was also further diluted in the supplemented medium with 20 µg/mL gentamycin and centrifuged into contact with the *Chlamydia*-infected monolayer as described above. After infection, all monolayers were washed with PBS twice and incubated for 12 hours in a supplemented medium. For the gentamycin *N. gonorrhoeae* wells, the supplemented medium also contained 20 µg/mL gentamycin. The co-infection experiment was harvested and enumerated as previously described.

Tryptophan and indole recovery assay

The co-infection experiment was set up as previously described. After centrifuging *N. gonorrhoeae* into contact with the monolayer and washing non-adherent bacteria away, the medium was replaced with a supplemented medium containing 10 µM indole or 400 µM L-tryptophan. *C. trachomatis* mono-infections were also incubated in a supplemented medium containing 10 µM indole or 400 µM L-tryptophan. At 12 hpci, all wells were harvested and enumerated as previously described.

Co-infection in transwell plates

The co-infection assay was performed as previously described with the exception that 0.4 µm transwells were placed above *C. trachomatis*-infected monolayers. The transwells contained 150 µL of *N. gonorrhoeae* at approximately 6.7×10^9 CFU/mL or 150 µL of supplemented medium. At 12 hpci, the transwells were carefully removed and all bacteria enumerated as described above.

RNA purification, reverse transcription, and qPCR for gene expression

The co-infection was performed as previously described. Infected monolayers were resuspended into 150 µL of PBS and 750 µL RNA. Later, reagents were added for preservation. Total RNA was extracted using the Direct-Zol RNA miniprep kit (Zymo) including the on column DNaseI treatment step, according to the manufacturer's instructions. A second DNA removal step was performed using TURBO DNase (Invitrogen). RNA concentration was measured using a Nanodrop spectrophotometer (Fisher Scientific) and gDNA removal was validated using qPCR (primers u_16S F and u_16S R). cDNA was synthesized using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems) and a no reverse transcriptase (NRT) control was included for each sample. cDNA was diluted 1:5 in molecular biology water before real-time amplification on a CFX 96 thermocycler (BioRad) using Luna Universal qPCR Mastermix (New England Biolabs). Primer combinations for each gene target are listed in Table S1. Chlamydial genomic DNA was used to prepare a standard curve for calculating transcript copy number and all target genes were normalized to 16S transcript levels.

Statistical analysis

Data sets with three or more groups were analyzed by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons for the post hoc test. Data sets with less than three groups were analyzed using an unpaired Student's *t*-test. *P* values of ≤ 0.05 are considered statistically significant. Graphpad Prism was used for all calculations.

ACKNOWLEDGMENTS

The authors would like to acknowledge the members of the Maurelli and Liechti labs for their helpful discussions. We also thank Drs. Alison Criss, Joseph Dillard, and Cory Leonard for their advice, discussions, and reagents with growing Neisseria gonorrhoeae.

Funding support to L.M.B. was provided by the Graduate Program for Biomedical Sciences at the University of Florida, and the Department of Environmental and Global Health at the University of Florida. Funding support to G.W.L. was provided via a MIRA ESI award from NIGMS (GM138202).

G.W.L. and E.B. The opinions or assertions contained herein are ours and are not to be construed as official or as reflecting the views of the Department of Defense or the Uniformed Services University.

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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Figure S1 (IAI00179-23-s0001.TIF). Modified co-infection timeline.
Figure S2 (IAI00179-23-s0002.TIF). *Chlamydia muridarum* co-infection with *N. gonorrhoeae*.
Supplemental material legends (IAI00179-23-s0003.docx). Legends for Fig. S1 and S2 and Table S1.
Table S1 (IAI00179-23-s0004.docx). Primers used in this study.

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