IMMUNITY TO NEISSERIA GONORRHOEAE

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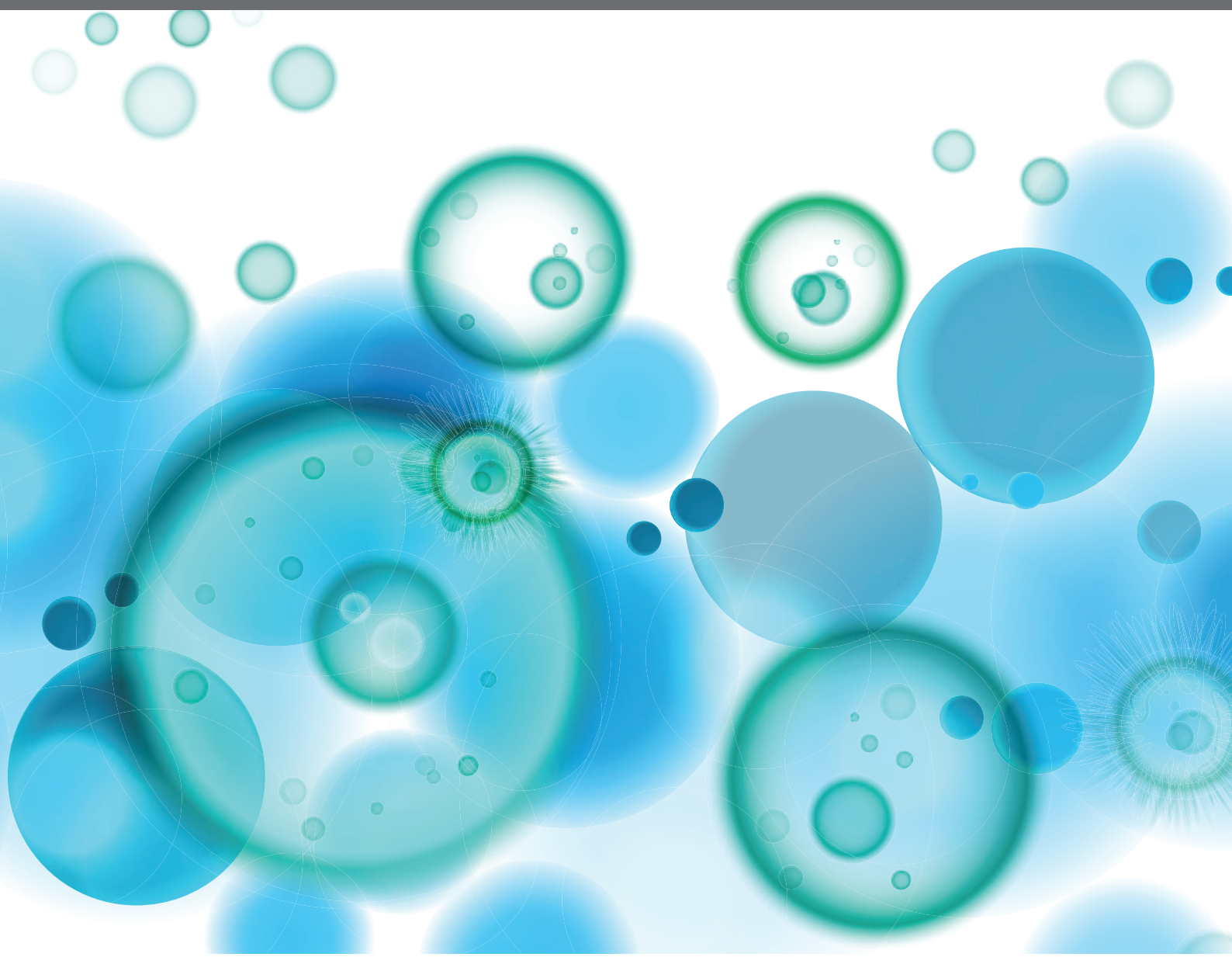

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IMMUNITY TO NEISSERIA GONORRHOEAE

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Editorial: Immunity to *Neisseria gonorrhoeae*

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Editorial on the Research Topic

Immunity to *Neisseria gonorrhoeae*

Immunity to *Neisseria gonorrhoeae* has been hard to define because, until recently, there has been no evident state of immunity to gonorrhea in humans; re-infections are fairly common, implying that an episode of infection does not usually induce a protective immune response. Experimental investigation is complicated by the fact that humans are the only known natural host for this infection. However, recent experimental findings using a variety of *in vitro* and *in vivo* model systems, in addition to clinical and epidemiological studies, have begun to cast new light on this problem. This Research Topic brings together 12 papers from leading contributors to the field, affording a comprehensive overview of current understanding and suggesting future pathways of investigation that should lead to actionable understanding of the immuno-pathogenesis of gonococcal infection, as well as facilitate novel approaches to therapeutic treatment and the development of a prophylactic vaccine. The need for such innovations is highlighted by the ongoing emergence of resistance in *N. gonorrhoeae* to all currently available antibiotics, which threatens to render gonorrhea untreatable (1), as well as the unacceptably high prevalence of gonorrhea. Global incidence is now estimated at 87 million new infections every year (2), with the heaviest burden of reproductive tract morbidity falling on women, especially in low- and middle-income countries and underserved populations.

The articles in this Research Topic fall broadly into two connected threads: human immune responses to gonococcal infection; and gonococcal vaccine development. First, Lovett and Duncan set the scene with a discussion of what is currently known about the human immune response to *N. gonorrhoeae* and the natural history of the infection. Crucially, many cervical and pharyngeal infections are "asymptomatic," meaning that the subject is unaware of her or his infection. This vitiates the evaluation of host responses that typically develop with the onset of inflammation and profuse exudation of neutrophils. Further complications in studying immune responses arise because of the antigenic diversity of *N. gonorrhoeae* as well as similarity to commensal *Neisseria* species that frequently occur in the oropharynx. Antibody responses remain weak until upper tract infection ensues, but, by that stage, inflammatory damage develops with potentially serious consequences such as scarring of the epididymis or Fallopian tubes, which can lead to infertility in both sexes, and ectopic pregnancy and pelvic inflammatory disease in women. The pathogenesis of upper tract infection and the associated host responses are reviewed by Lenz and Dillard, focusing especially on the use of human tissue explants, which complements findings derived from animal models in which infection is unnatural.

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Gonococcal infection enhances the transmission and acquisition of HIV, and the mechanisms of this poorly understood interaction are discussed by Guvenc et al., revealing multifactorial, bidirectional effects. Mechanisms include the ability of *N. gonorrhoeae* to selectively induce Th17 responses and concomitantly suppress Th1/2-driven adaptive immune responses through several pathways, compromising immune functionality and increasing the primary target Th17 cells for HIV. In addition, the NFκB-dependent pro-inflammatory effects of several gonococcal components can promote HIV transcription, while gonococcal interactions with different mucosal antigen-presenting cells also appear to either enhance or oppose viral replication and immunity. The prevalence of *N. gonorrhoeae* means that these interactions have significant impact on the ongoing HIV pandemic.

The profuse exudation of neutrophils into the male and female genital tracts characterizes symptomatic infection and constitutes a classical diagnostic criterion, yet apparently intact gonococci are frequently observed within these phagocytes on microscopic examination of exudate smears. Several mechanisms have been elucidated whereby *N. gonorrhoeae* resists intracellular destruction within neutrophils (3), and here Handing et al. demonstrate the role of the MtrCDE efflux pump, which has previously been recognized to be important in the resistance of *N. gonorrhoeae* to host antimicrobial peptides and antibiotics (4, 5), in avoidance of neutrophil killing. While neutrophils are the prime responders during gonorrhea, the singular focus on neutrophils has detracted from considering the role of other "professional phagocytes," i.e., macrophages, which typically follow the influx of neutrophils in pyogenic infections. Although the presence of macrophages has been observed in the female mouse model of gonococcal infection (6), few studies have addressed their role. The review by Escobar et al. draws attention to the ability of *N. gonorrhoeae* to modulate macrophage differentiation into the "alternative" M2 pathway, and the consequences of this for gonococcal survival. Therapeutic strategies aimed at reversing this macrophage polarization, such as by use of COX-2 inhibitors, might have a beneficial effect.

Nutritional immunity, defined as the prevention of infection through deprivation of essential nutrients, is well-exemplified by the with-holding of metals such as iron and zinc by means of high-affinity chelating proteins. Pathogenic Neisseria express several proteins to acquire these metals from the host, the best known being the transferrin- and lactoferrin-binding proteins. Yadav et al. review the molecular structures of these and other metal-acquiring proteins, thereby illustrating their mechanisms of action.

Interest in developing vaccines against *N. gonorrhoeae* has been reinvigorated by the emergence of multidrug resistance, as well as the recent findings concerning the immuno-pathogenesis of gonococcal infection and the response to it, as reviewed by Russell et al.. A landmark paper by Petousis-Harris et al. (7) reported that recipients of a meningococcal vaccine (MeNZB), based on outer membrane vesicles (OMV) from a group B strain of *N. meningitidis*, were 31% less likely to be diagnosed later with gonorrhea than unimmunized subjects. This represented the first report of a state of protective immunity against gonorrhea in humans. Petousis-Harris and Radcliff review this and subsequent studies, and discuss potential mechanisms underlying cross-protection, including the possible induction of antibodies against shared antigens. OMV have the advantage of combining multiple individual antigens, including proteins as well as lipo-oligosaccharide, in one vaccine, which might explain the cross-protection of MeNZB against gonorrhea in humans, as well as the effectiveness of an experimental gonococcal OMV vaccine against antigenically diverse strains of *N. gonorrhoeae* in mice (8). However, numerous defined antigens have also been proposed as potential vaccine candidates [(9, 10), Russell et al.]. Reverse vaccinology has been successful in identifying several proteins for inclusion in the recently licensed meningococcal vaccine, Bexsero® (marketed by GlaxoSmithKline), and the use of this approach combined with immuno-proteomics and bioinformatics studies to identify novel gonococcal antigens is reviewed by Baarda et al.. More classical microbiological approaches have led to promising efforts to target a lipo-oligosaccharide epitope, designated 2C7, which is present in >95% of *N. gonorrhoeae* strains. Gonococcal lipo-oligosaccharide itself is highly reactogenic and contains numerous other epitopes that are variably expressed by different isolates, but an alternative approach focusing on 2C7 has been developed by means of peptide mimics, as discussed by Gulati et al.. Because expression of transferrin-binding proteins is required for human (male) infection by *N. gonorrhoeae* (11), they have been proposed as potential vaccine antigens. To overcome limitations imposed by their variable antigenic structure and other factors, Fegan et al. have created hybrids of transferrin-binding proteins A and B to use as vaccine immunogens capable of inducing bactericidal antibodies. Finally, Jen et al. describe gonococcal methionine sulfoxide reductase, which is required for gonococcal resistance to oxidative stress, as a novel vaccine candidate.

Thus, the stage is now set for significant advances in comprehending immunity to *N. gonorrhoeae* and in developing an effective vaccine against gonorrhea. The contributions to this Research Topic reveal a remarkable range of experimental models and technologies that have been brought to bear upon solving these problems. What is now required is translation of experimental findings to the human disease, with the objective of delivering novel interventions for the treatment and prevention of gonococcal disease and its devastating sequelae.

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MR wrote the first draft of this editorial. All authors reviewed and edited the final document.

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The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Human Immune Responses and the Natural History of *Neisseria gonorrhoeae* Infection

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The intimate relationship between humans and *Neisseria gonorrhoeae* infections span centuries, which is evidenced in case reports from studies dating back to the late 1700s and historical references that predate medical literature. *N. gonorrhoeae* is an exclusive human pathogen that infects the genital tract of both men and women as well as other mucosal surfaces including the oropharynx and rectum. In symptomatic infections, *N. gonorrhoeae* induces a robust inflammatory response at the site of infection. However, infections can also present asymptomatically complicating efforts to reduce transmission. *N. gonorrhoeae* infections have been effectively treated with antibiotics since their use was introduced in humans. Despite the existence of effective antibiotic treatments, *N. gonorrhoeae* remains one of the most common sexually transmitted pathogens and antibiotic resistant strains have arisen that limit treatment options. Development of a vaccine to prevent infection is considered a critical element of controlling this pathogen. The efforts to generate an effective gonococcal vaccine is limited by our poor understanding of the natural immunologic responses to infection. It is largely accepted that natural protective immunity to *N. gonorrhoeae* infections in humans does not occur or is very rare. Previous studies of the natural history of infection as well as some of the humoral and cellular immune responses to infection offer a window into the issues surrounding *N. gonorrhoeae* vaccine development. In this review, we summarize the current body of knowledge pertaining to human immune responses to gonococcal infections and the role of these responses in mediating protection from *N. gonorrhoeae*.

Keywords: *Neisseria gonorrhoeae*, immune response, natural history, human infection, immunoglobulin, lymphocyte

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INTRODUCTION

Neisseria gonorrhoeae is a bacterial sexually transmitted pathogen that most commonly infects the lower genital tract, the cervix in women, and anterior urethra in men. *N. gonorrhoeae* can also infect other mucosal surfaces, particularly the pharynx and rectum. Symptoms associated with disease including purulent urethral or cervical discharge and discomfort at the site of infection are due to the pathogens ability to induce robust localized inflammation within the host. However, asymptomatic infections with *N. gonorrhoeae* are common and may serve as a significant reservoir of transmissible bacteria in the population. In women, untreated infections can ascend to the upper genital tract leading to a number of health complications including pelvic inflammatory disease

and infertility. Rarely, infection with *N. gonorrhoeae* disseminates leading to septic arthritis and skin manifestations. Antibiotic resistance in *N. gonorrhoeae* strains is on the rise worldwide and effective treatment options have become limited. Although individuals with gonococcal infections are known to produce anti-gonococcal humoral immune responses, it is clear that most of these responses are insufficient for providing protection from future infection. We will review the current state of knowledge regarding immune responses to gonococcal infections and the role of those responses in mediating protection from *N. gonorrhoeae* in humans. Better understanding of the immune response to natural infection with *N. gonorrhoeae* is vital for the prevention of disease transmission and the development of an effective gonococcal vaccine.

NATURAL HISTORY OF *NEISSERIA GONORRHOEAE* INFECTION

Our understanding of the natural history of *N. gonorrhoeae* infection is hampered by a lack of rigorous scientific studies of microbiologically defined *N. gonorrhoeae* infection from the pre-antibiotic era. Since the introduction of sulfa-based antibiotics and subsequently penicillin, antibiotic treatment for men with symptomatic *N. gonorrhoeae* infection, usually urethritis, has been the standard of care. In studies of men seeking care for gonococcal urethritis subjects reported average incubation periods of ∼6–8 days between presumed exposure and onset of symptoms (1, 2). However, some individuals reported symptom onset as early as 1–2 days. These studies also indicated that men with symptomatic gonococcal urethritis were symptomatic on average for 7 days before seeking care, though that time ranged from 1 day to 1 year. Because current guidelines indicate men with symptomatic gonorrhea should be treated with antibiotics, there are no prospective studies describing natural clearance or natural progression of symptomatic infection. However, some information about the persistence of symptomatic *N. gonorrhoeae* infection can be drawn from treatment failures in therapeutic trials of antibiotics for gonococcal urethritis. Svinland et al. examined bacterial clearance after treatment with flumequine in 239 patients with uncomplicated gonorrhea (3). Although multiple dosing regimens with flumequine were effective at curing the vast majority of patients, there was a small number of patients who failed to clear the infection following treatment. *N. gonorrhoeae* infection was found to persist at the test of cure obtained after 14 days in 10 subjects. Six of those subjects harbored strains with high level flumequine resistance. Those six strains represented all high-level resistant strains found in the study and therefore represented a complete cohort of subjects who received ineffective antibiotic therapy in the study. The persistence of infection in all of these subjects suggests that a large proportion of symptomatic *N. gonorrhoeae* infections that go without treatment are likely to persist at least 14 days. Treatment failures have also been reported in a number of other therapeutic trials that also support the hypothesis that *N. gonorrhoeae* can infect the lower genital tract and persist in the face of localized inflammatory response for at least 14 days (4, 5). To characterize the average bacterial load during infection, Isbey et al. analyzed the urine and semen of men with symptomatic urethritis.

Since the treatment of men with asymptomatic gonorrhea has not always been the standard for clinical management of *N. gonorrhoeae* infection, Handsfield and colleagues conducted a prospective study of the natural history of asymptomatic male infection in the early 1970's. Asymptomatic men were identified via positive *N. gonorrhoeae* urethral cultures from men requesting STI screening or men that were contacts of women positive for symptomatic gonorrhea at Seattle STD clinics. Of the 28 patients examined weekly, 18 remained asymptomatic until they were treated, which varied from 7 to 165 days. Of the remaining 10 subjects, 5 developed urethritis, and the other 5 spontaneously cleared the infection (6). The determinants of symptomatic vs. asymptomatic infection remain unknown. The total number of *N. gonorrhoeae* recovered per urine sample ($\sim 6*10^6$ CFUs) and from semen ($\sim 7*10^6$ CFUs) suggests that *N. gonorrhoeae* are carried and/or excreted at large quantities during male infection (7). Experimental gonococcal infection with male volunteers mimics much of the clinical features of naturally acquired infections and has provided some insight on the natural history of early symptomatic infection. Infected subjects often develop dysuria and urethritis with the onset of symptoms ranging from ∼1 to 6 days post inoculation and *N. gonorrhoeae* can be recovered from the urine in as little as 2 h following inoculation. The quantity of bacteria recovered ($\sim 10^2$-10^5 CFUs/sample) does not appear to correlate with the severity of infection symptoms (8). Overall, these studies highlight the ability of *N. gonorrhoeae* to persist for prolonged periods in both symptomatic and asymptomatic men. Additionally, it is clear that asymptomatic infection can progress to symptomatic infection across a broad time spectrum. The determination of the natural rate of clearance of infection is complicated by the need to treat infected individuals with effective antibiotics.

Studies of the natural history of *N. gonorrhoeae* infection in women are also limited due to a standard of care that requires the use of antibiotics to treat known *N. gonorrhoeae* infection. In a case survey comparing cure rates of *N. gonorrhoeae* infection after treatment with sulfathiazole or penicillin, patients were followed for 3 months before being declared cured. The majority of the observed patients were female in this report. Sulfathiazole treatment had a 21% failure rate, with some patients found to have positive cultures 3 months or longer after initial therapy (9). A study of women determined to be recently exposed (average of 11 days after exposure) to *N. gonorrhoeae* provided some insight into the acquisition and presentation of *N. gonorrhoeae* infection. Twenty-six women were identified through contact tracing of partners of men with gonococcal urethritis. Of the 26 subjects, 19 were found to be infected with *N. gonorrhoeae*. Risk of infection after exposure in these women increased with number of exposures to the infection (sexual encounters with the infected contact): 6/12 women with one exposure were found to be infected while 6/7 women with two exposures and 7/7 women with more than two exposures were found to be infected. Of the 19 infected

subjects, 9 subjects had clinically defined pelvic inflammatory disease or tenderness of the adnexa suggestive of inflammation in the upper reproductive tract (10). Because of the risk for ascending infection, study of the natural history of asymptomatic infection in women, once identified is not considered ethically acceptable. However, Stupiansky et al. performed a study using self-collected vaginal swabs samples collected in a prospective cohort designed to study sexual health in adolescent women. In this study, subjects underwent examination and clinic-based STD screening every 3 months, 4 times more frequently than recommended for asymptomatic adolescent women. In between screenings, the subjects self-collected cervicovaginal swabs and kept diaries of sexual activity and urogenital symptoms. *N. gonorrhoeae* DNA was identified in cervicovaginal samples collected prior to the identification of *N. gonorrhoeae* infection at a quarterly visit in 18 women. Although the quantity of *N. gonorrhoeae* DNA found in self-collected swabs from individual women varied greatly, the mean bacterial load ($\sim 10^3$-10^5 CFUs/sample) was similar regardless of the length of time of infection. Additionally, women with *Chlamydia trachomatis* coinfections displayed higher mean bacterial load, though the difference in *N. gonorrhoeae* DNA levels between *C. trachomatis* co-infected and uninfected subjects was not statistically significant. Because the longest period between clinic-based STD screens in this was 12 weeks, persistent infection longer than 12 weeks could not be identified in this group. Interestingly, vaginal discharge and dysuria were reported in the diaries of 3 of the 18 women. However, the presence of symptoms did not correlate with bacterial load (11). These studies suggest that both symptomatic and asymptomatic *N. gonorrhoeae* infections can persist in women for at least 12 weeks. Further, the frequency of natural clearance in the setting of prolonged infection was not reported for these 18 subjects, but it at least some infections persisted from initial onset up to the quarterly in person visit. Taken together with evidence of persistent symptomatic and asymptomatic infections in men, this report suggests that *N. gonorrhoeae* is capable of evading or resisting host immune responses to infection.

Historical writings like those from James Boswell, an eighteenth century biographer who reported 20 episodes of symptoms consistent with gonococcal infection, have long been offered as anecdotal evidence that *N. gonorrhoeae* infection does not induce protective immunity (12). Studies that provide insight into the natural history of *N. gonorrhoeae* infection suggest that prior infection with *N. gonorrhoeae* induces little protective immune responses to the pathogen. Platt's study of *N. gonorrhoeae* acquisition by women exposed to men with gonococcal urethritis demonstrated that 1–7 women exposed and uninfected had prior history of *N. gonorrhoeae* infection while 10 of 19 exposed and infected individuals had prior history of *N. gonorrhoeae*, which is consistent with prior infection not leading to protective immune response (10). An epidemiologic study of *N. gonorrhoeae* infections in rural North Carolina found 14.8% of *N. gonorrhoeae* infected individuals experienced a second infection during the study period. The *N. gonorrhoeae* strain recovered from those repeatedly infected individuals was more likely to be the same serovar as the strain recovered from their initial infection than an alternative serovar, suggesting that strain specific immune responses were inadequate to provide protection from *N. gonorrhoeae* (13). In the prospective cohort study of adolescent women that described the presence of *N. gonorrhoeae* DNA in self-collected cervicovaginal swabs, no difference in the quantity of *N. gonorrhoeae* DNA was observed between individuals with a history of prior infection (11). Overall, the data from clinical studies of *N. gonorrhoeae* infection suggest that immune clearance of infection and protection from repeated infection are largely inadequate leading to prolonged symptomatic and asymptomatic infections (**Figure 1**).

HUMORAL IMMUNE RESPONSES TO *N. GONORRHOEAE* INFECTION (TABLE 1)

Individuals infected with *N. gonorrhoeae* have been shown to produce anti-gonococcal antibodies in sera, seminal plasma, and cervical secretions. Although increases in the different immunoglobulin classes are detected in the serum and secretions of infected individuals, the difference between infected and uninfected individuals have often been reported as modest. Hedges et al. tested both serum and secretions of infected and uninfected male and female attendees of an STD clinic for antibodies against the MS11 strain and homologous infecting strains. When compared to uninfected patients, slight increases in serum IgA1 from female patients and serum IgG from male patients against the MS11 strain were observed. The level of serum IgA and IgG against infecting isolates did not drastically change over the 6-week observation period. Additionally, no difference in anti-gonococcal antibody levels was noted between individuals with no prior history of infection and previously infected individuals regardless of their current infection status (20). However, this study focused on antibody directed against fixed whole bacteria and not specific antigens, suggesting that the bulk of the reactive antibodies measured in this assay resulted from cross reactive immunoglobulins directed against commensal bacterial targets or that the assay was performed in a way that detected high level non-specific antibody binding that masked the ability to detect specific immune responses to gonococcal infection. Antigen specific antibody responses have been reported for a number of gonococcal polysaccharide and protein antigens, in some studies preadsorption of sera with other bacterial species may have contributed to the ability to detect antibodies that developed specifically during *N. gonorrhoeae* infection.

Polysaccharides are a major source of microbial antigens from pathogenic Neisseria species, as well as many other bacteria. Unlike *Neisseria meningitidis*, *N. gonorrhoeae* does not produce a polysaccharide capsule, which is a major immunogenic, saccharide-based antigen for *N. meningitidis*. However, both *N. gonorrhoeae* and *N. meningitidis* produce lipooligosaccharide (LOS) which makes up roughly 50% of the mass of the outer membrane of the bacteria. LOS from *N. gonorrhoeae* and *N. meningitidis* share a common core oligosaccharide along with lipid A structure (21). The structure of the oligosaccharide of LOS is determined by the expression of multiple phase variable

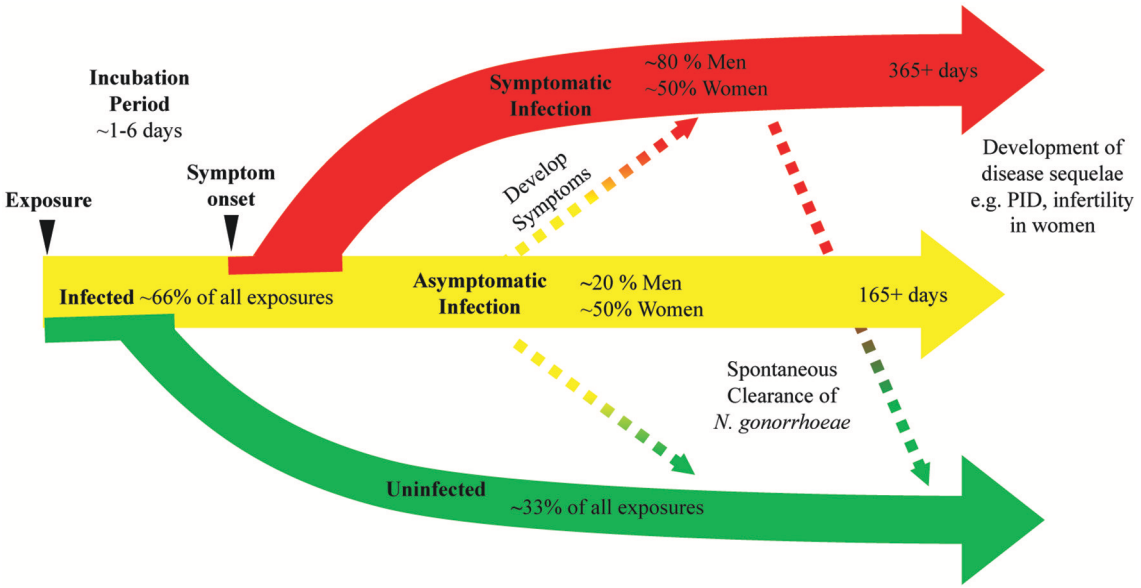

FIGURE 1 | *N. gonorrhoeae* exposure can lead to bacterial clearance or prolonged infection with or without symptoms. A diagram of the natural history of *N. gonorrhoeae* infection after exposure demonstrates as many as 33% of exposed individuals will not develop infection. Infected individuals can have symptoms or remain asymptomatic. Asymptomatic infection can eventually progress to symptomatic infection, with studies indicating this progression may occur in as many as 25% of asymptomatic infections. Symptomatic infection can persist at least 14 days and infection is documented to last as long as 1 year. Asymptomatic infection is documented to persist as long as 165 days with as many as 25% of asymptomatic infections clearing over that time frame. The use of antibiotic therapy in all studies generating these data limit our knowledge of the actual rates of bacterial clearance and length of time chronic infection can persist.

TABLE 1 | Anti-gonococcal immunoglobulins identified in humans.

Antigen	LOS[a,b,c]	Pili[a,d]	Protein I (PorB)[a]	Protein II (Opa)[a]	Protein III (Rmp)[a]	Lip[a]	Tbp[f]	Whole Bacteria[g]
Pre-existing Antibody (or present in healthy controls)	+	+	+	+	+	+	+	+
Post-infection Antibody	++	++	+	+	+	+	+	+
Immunoblot/ELISA	I/E	E	I	I	I	I	E	E

[a]*Hicks et al. (14);* [b]*Schmidt et al. (15);* [c]*Yamasaki et al. (16);* [d]*Meittenen et al. (17);* [e]*Plummer et al. (18);* [f]*Price et al. (19);* [g]*Hedges et al. (20).*

saccharide transferases (22). Thus, even within a single strain there can be substantial LOS heterogeneity. The ability of these variations in structure to lead to heterogeneity in antigenic epitopes that can be recognized by antibodies was demonstrated clearly by the characterization of *N. gonorrhoeae* LOS from 20 different strains with a panel of murine monoclonal antibodies (23). Despite these variations in LOS structure, almost all *N. gonorrhoeae* strains examined by Gulati and colleagues maintain expression of the LOS structure recognized by monoclonal antibody 2C7 (24). Antibodies to LOS have been detected in both normal human sera and in sera from *N. gonorrhoeae* infected individuals. Sera collected from a small subset of male subjects in a gonococcal pilus-based vaccine trial were used to characterize antigen specific antibody responses during acute gonococcal infection. These subjects who received the placebo vaccination, reported no previous history of STIs, had a negative culture at the start of the trial and developed gonococcal urethritis during the trial period. Sera were collected bi-weekly over the course of 8 weeks. This study found 4 of 13 subjects carried pre-existing LOS antibodies that presumably arose from cross reactivity with polysaccharides from other commensal bacteria species. Of the remaining 9 subjects who did not have preexisting antibodies that recognize *N. gonorrhoeae* LOS, 6 developed LOS antibodies after developing *N. gonorrhoeae* infection (14). In another study, sera collected from patients with complicated gonococcal infections [8 disseminated gonococcal infections, 4 cases of pelvic inflammatory disease (PID), and 1 case of epididymitis] was used to characterize antibody responses to *N. gonorrhoeae* outer membrane proteins. Sera obtained from a majority of patients (9/13) exhibited antibody responses to LOS (4). Antibodies against LOS were also studied by researchers using an experimental human male urethral inoculation to test whether recent gonococcal urethritis led to protection from reinfection. Some immunoreactivity toward *N. gonorrhoeae* LOS was detected in serum from 21 of 24 subjects prior to inoculation with *N. gonorrhoeae* for the first time. Of the 14 subjects in the

study who were rechallenged, 6 of 14 demonstrated an increase in anti-LOS antibody demonstrated by immunoblot, and 9 of 14 had at least a 2-fold increase in antibody titer measured by ELISA (15). Consistent with baseline observations of subjects in these studies, the presence of *N. gonorrhoeae* LOS-directed antibodies was detected from pooled sera taken from eight volunteers with no history of gonococcal infection (16). The predominant antibodies that bound LOS in this study were of the IgG class. Affinity purified anti-oligosaccharide IgG isolated from normal human serum was found to contain bactericidal activity toward a serum sensitive strain of *N. gonorrhoeae*. In a separate study, Yamasaki and colleagues found that *N. gonorrhoeae* LOS-binding IgG2 from NHS recognized at least 3 different oligosaccharide (25). Thus, numerous studies indicate that the majority, but not all, humans mount antibody responses to a variety of saccharide epitopes found in the lipooligosaccharide of *N. gonorrhoeae* in response to mucosal or invasive infection with the bacteria.

A number of studies have surveyed whether different gonococcal protein antigens induce humoral immune responses during infection. Sera isolated from 68 patients with uncomplicated and 35 women with PID were used to characterize antibody responses to gonococcal pili (17). The mean antibody levels of IgG, IgA, and IgM against *N. gonorrhoeae* pilus was significantly higher in men with urethritis (∼2-fold increase) and women with cervicitis (∼4-fold increase) when compared to sera from uninfected patients. Anti-pilus antibodies were also significantly higher in sera from women with confirmed gonococcal PID when compared to sera from patients with non-gonococcal PID. In addition to testing for anti-LOS antibodies, sera isolated from male volunteers participating in pilus vaccine trial who acquired gonorrhea and who reported no previous history of gonococcal infection were analyzed for the presence on anti-gonococcal protein antibodies using immunoblot analysis. Pre-existing antibodies were detected in 12 of 13 subjects by western blot analysis using pre-infection serum. Not only were preexisting antibodies against LOS detected but antibodies against a number of abundant outer-membrane proteins were observed including: Protein I (PorB), Protein III (Rmp), Pili, and Lip. The authors suggested that the preexisting antibodies to LOS and *N. gonorrhoeae* proteins may be a result of cross reactivity to antigens from nasal carriage of *N. meningitidis*. After acquisition of *N. gonorrhoeae* infection, 9 of 13 subjects developed IgG serum antibody responses with antibodies against all 6 major outer membrane antigens analyzed in this study (LOS as well as Proteins I, II, III, Pili, and Lip). In the aforementioned study by Hook et al. in which sera collected from 8 patients with complicated *N. gonorrhoeae* infections, Protein I antibodies were found in sera collected from all 13 patients with complicated gonococcal infections (4). Antibodies against both PorB (Protein I) and Opa (Protein II) were also detected in the sera of almost all commercial sex workers in Nairobi, Kenya, that were enrolled in a prospective cohort study of sexually transmitted infections (18, 26). The combination of increased levels of gonococcal antigen-directed antibodies in infected humans combined with prevalent antibodies in a highly exposed cohort, suggest that infection with *N. gonorrhoeae* can cause the development of antibodies directed toward the pathogen. A study of antibody titer or development of specific pathogen-directed antibodies during prolonged infection or after resolution of infection would be required to demonstrate the antibodies develop in response to infection.

Immune responses to gonococcal transferrin binding proteins were analyzed by ELISA using sera and secretions collected from patients attending STD clinics who had confirmed positive *N. gonorrhoeae* cultures. Sera from healthy volunteers were used as controls. Antibodies to both transferrin binding proteins, TbpA and TbpB, were detected in sera from men and women. However, only IgA and IgM concentrations against TbpB in women was significantly higher than the levels of anti-TBP antibodies in uninfected control sera. One male subject's serum antibody levels to TbpA and TbpB were followed for 6 months. A slight increase in IgG, IgM, and IgA was observed 1 month after infection and the levels returned to baseline for the following 5 months of the study, suggesting that the antibody titers increased in response to infection (19). In the previously mentioned two phase study utilizing urethral challenge and rechallenge, with *N. gonorrhoeae* in male volunteers with no previous history of gonococcal infection, pre-infection sera from each volunteer contained IgG to at least one of the major gonococcal Outer Membrane Proteins. A majority (18/24) of the volunteers had pre-existing anti-pilus antibodies. Immunoblots conducted with pre- and post-infection sera were reported from a subset of these subjects. Increases in sera antibodies to outer membrane proteins, particularly IgG, was detected following infection in most subjects however the pattern of recognized proteins varied from subject to subject. Though *N. gonorrhoeae* exposure or infection prior to enrollment was thought to explain the preexisting detectable antibodies to *N. gonorrhoeae* antigens in the Kenyan commercial sex-worker cohort study, the number of different serogroups of PorB recognized by sera from subjects in this cohort rose with the number of study visits, suggesting that ongoing exposure to new strains of *N. gonorrhoeae* was resulting in additional antibody responses to that gonococcal antigen (26). Overall, these studies also support the finding that as with lipooligosaccharide, pre-existing antibodies to gonococcal protein antigens are common in humans but antibodies to some antigens do appear to increase in response to infection.

While there is clear *in vitro* evidence that some antibodies directed toward *N. gonorrhoeae* antigens can promote either complement mediated killing or opsonophagocytic killing of the bacteria, whether these antibodies can prevent human infection or play a role in immunologic clearance of the pathogen remains an open question. In a study of experimental human infection followed by rechallenge with *N. gonorrhoeae*, 7 of 8 subjects who experienced at least a 2-fold increase in anti-LOS antibody resisted reinfection while only 1 of the 6 subjects who had less than a 2-fold increase in anti LOS were susceptible to reinfection (15). Many human IgG and mouse monoclonal antibodies that recognize *N. gonorrhoeae* LOS epitopes are bactericidal (16, 27). Passive immunization with an antibody directed against the common 2c7 epitopes of *N. gonorrhoeae* is protective in a mouse model of vaginal *N. gonorrhoeae* infection (28). In a prospective study of a cohort of commercial sex

workers in Kenya, a strong association between the number of different serogroups of *N. gonorrhoeae* Opa protein (protein II) recognized by antibodies in subject sera and reduced relative risk of gonococcal salpingitis was observed (18). However, in the same cohort, detectable antibody to Rmp (protein III) was associated with increased risk of gonococcal infection during the study as well as increased rate of gonococcal salpingitis (26). Although antibodies against Rmp are detected during infection, there is substantial evidence indicating that many of the antibodies against *N. gonorrhoeae* Rmp can block bactericidal activity of other *N. gonorrhoeae*-directed antibodies. Serum isolated from patients with disseminated gonococcal infection was shown interfere with *in vitro* killing of *N. gonorrhoeae* by normal human sera. Early studies aimed at identifying the mediators responsible for this blocking of normal human sera bactericidal activity demonstrated that IgG was the major source of blocking activity in sera from subjects with disseminated gonococcal infection (29, 30). IgG reactive with gonococcal outer membrane proteins was more effective at inhibiting bactericidal activity when compared to normal IgG and depletion of Outer Membrane Protein-binding IgG restored the bactericidal activity of serum. Further examination into specific antigens that elicit blocking antibody responses found that RMP-binding IgG contributed to the majority of the blocking activity observed *in vitro* (31). Price and colleagues were able to demonstrate that by preincubating *N. gonorrhoeae* with Rmp-directed IgG from immune sera decreased the binding of bactericidal antibodies from normal human sera to *N. gonorrhoeae* and inhibited killing of the bacteria by normal human sera (31, 32). Some mouse monoclonal antibodies against *N. gonorrhoeae* Rmp generated through immunization have been found to have bactericidal activity while others have been shown to have blocking activity similar to that of human sera containing anti-Rmp IgG. Analysis of two Rmp-directed monoclonal antibodies with blocking activity revealed they ound an overlapping linear epitope of the protein between amino acids 24–33, an epitope with substantial homology to an outermembrane protein from *Escherichia coli*, OmpA (33–35). Another study examining the blocking epitope of Rmp bound by antibodies in sera from humans with disseminated gonococcal infection demonstrated that antibodies directed to residues in disulfide-containing loop near the OmpA homologous region (amino acids 47–64) also had blocking activity (36). In a recent study, Gulati et al. demonstrated that passive immunization with an anti-Rmp monoclonal antibody abrogated the protective effects of the 2c7 monoclonal antibody administration in a female mouse *N. gonorrhoeae* infection model, causing both increased bacterial burden and duration of infection (28, 37). Although the Rmp protein is conserved and immunogenic, the production of anti-Rmp antibodies can inhibit the development of protective humoral responses. Overall, despite clear evidence that humans develop anti-gonococcal antibodies in response to infection, repeat infections with *N. gonorrhoeae* are common. These data indicate that the humoral immune responses induced by *N. gonorrhoeae* infection are complex and in most cases are insufficient to provide protection from infection.

CELL MEDIATED IMMUNE RESPONSES TO *N. GONORRHOEAE* (TABLE 2)

In addition to humoral immune responses to *N. gonorrhoeae*, there is also evidence that humans with *N. gonorrhoeae* develop adaptive cellular immune responses specific to *N. gonorrhoeae* antigens during infection. Early studies of *N. gonorrhoeae* cellular immunity were conducted by measuring proliferation in response to antigen stimulation as demonstrated by radioactive adenine incorporation into DNA in cultured primary lymphocytes. Mauss and colleagues studied proliferation of lymphocytes obtained from patients with *N. gonorrhoeae* infection as well as a set uninfected male controls in response to a variety of *N. gonorrhoeae* antigen preparations. For the best characterized antigen preparation 3 of 6 female subjects and 4/4 male subjects with *N. gonorrhoeae* infection had a positive proliferative response while 1 of 7 uninfected men had a positive response (9). Two similar but larger and better controlled studies confirmed these findings, Kraus and colleagues studied lymphocyte proliferation in response to gonococcal antigens in men with gonorrhea and control male subjects with no reported history of gonorrhea (40). Lymphocyte proliferation was higher in lymphocytes from men with two or more gonococcal infections while lymphocytes from those subjects with their initial case of gonorrhea did not exhibit antigen induced proliferation. Similarly, Wyle et al. found that 21 women and 29 men with culture proven *N. gonorrhoeae* infection were found to have significantly higher proliferative indexes in response to *N. gonorrhoeae* antigen than lymphocytes from uninfected men and women who denied prior history of *N. gonorrhoeae* infection (41). Proliferative responses observed in lymphocytes from *N. gonorrhoeae* infected individuals fell below the level of detection in most individuals within 5 weeks of treatment (43). The specific *N. gonorrhoeae* antigens that are recognized by cellular immune responses in humans are poorly studied. One study demonstrated that the abundant membrane protein PorB could provide antigenic stimulation to lymphocytes from 20 and 24 of 30 *N. gonorrhoeae* infected subjects (38). In that study, a lack of PorB-induced proliferation was reported for lymphocytes from uninfected control subjects, suggesting PorB-directed lymphocytes that might arise from cross-reactive bacterial antigens are not highly represented in humans. In a separate study, lymphocytes from 8 of 8 healthy individuals demonstrated proliferative responses to treatment with *N. gonorrhoeae* IgA protease (39). It is possible these findings result from cross-reactive lymphocyte responses in lymphocytes that recognize homologous IgA proteases from commensal oral pharyngeal bacteria or even *N. meningitidis*. Genomic analysis of 4 gonococcal strains identified 23 conserved proteins with predicted T and B cell epitopes that could serve as universal antigens (44). However, the gonococcal antigens that elicit natural immune responses during infection remains largely unstudied, or at least unreported in the medical literature, at this time.

Lymphocytes can elicit a variety of functional responses that are aimed at clearing pathogens from the host upon

TABLE 2 | Anti-gonococcal cellular immune responses identified in humans.

Antigen	Protein I (PorB)[a]	IgA protease[b]	Whole Bacteria[c,d,e,f]
LYMPHOCYTE PROLIFERATION			
uninfected, pre-infected	−	+	+
infected	+	NT	++
STIMULATED CYTOKINE RELEASE: (CELL TYPE)			
IL-4	+ (CD4 & CD8 T cell)	+ (PBMC)	+ (PBMC)
IL-10	−	+ (PBMC)	+ (PBMC)
IFNγ	−	+ (CD4 T cell, PBMC)	+ (PBMC)
TNF-α	−	+ (PBMC)	NT

NT, not tested; CD4 or CD8 T cell, cytokine detected in T cell population by intra-cellular cytokine staining; PBMC, cytokine detected in Peripheral Blood Mononuclear Cell culture supernatant by ELISA. [a]Simpson et al. (38); [b]Tsirpouchtsidis et al. (39); [c]Kraus et al. (40); [d]Wyle et al. (41); [e]Mauss (9); [f]Rarick et al. (42).

recognition of pathogen-derived antigens. Differential functions of T lymphocytes are mediated by differential expression of a combination of cell surface proteins and secreted mediators (cytokines and chemokines). Lymphocytes responsible for coordinating immunologic responses known as T helper cells are positive for the CD4 antigen (CD4+) and are generally sub-classified into 4 broad groups that each have a predominant secreted cytokine: 1) Th1 which secrete interferon-gamma (IFNγ), Th2 which secrete interleukin-4 (IL-4), Th17 which secrete interleukin-17 (IL-17), and Treg which secrete interleukin-10 (IL-10). Mucosal infections typically do not induce profound differences in systemic or circulating levels of cytokines. However, sera isolated from patients with gonococcal infection were found to have modestly higher circulating levels of IL-17, IFNγ, and IL-23, when compared to sera from healthy controls (45). An inverse correlation between serum levels of IL-17 and serum levels of IFN-γ was observed suggesting that Th1 responses are blunted as Th17 responses are generated during *N. gonorrhoeae* infection. In a study of cervical immunologic factors in women undergoing STI screening at primary care clinics in Durbin, South Africa, IL-17 was found to elevated in cervical secretions of women with *N. gonorrhoeae* infection when compared to women without evidence of bacterial STI (46). IL-17 and other inflammatory cytokines (IL-1α, IL-1β, IL-12p70, TNF-α, RANTES, G-CSF, Flt3L, IL-2, IL-5, IL-15, and IL-17) were also found elevated in cervical lavage of *N. gonorrhoeae* infected women when compared to women with no detectable STI (47). In a study of women seeking care in a Nairobi STI clinic for acute abdominal pain or vaginal discharge, cervical IL-10, a cytokine produced by both regulatory T cells (Treg), and other immunoregulatory cells, was detected in 19 of 59 women with no detected bacterial STI and in 19 of 36 women with *N. gonorrhoeae* infection. Overall, published studies suggest that *N. gonorrhoeae* induces a complex lymphocyte response that is largely driven by an Il-17 or Th17 pro inflammatory response.

While there are a number of studies in which the level of cytokines in serum or site-specific fluids from *N. gonorrhoeae* infected individuals, few reported studies of the CD4+ T cell functional responses to *N. gonorrhoeae* in humans also exist. IgA protease-directed CD4 cells from healthy individuals without known *N. gonorrhoeae* infection have been shown to produce IFNγ using both elispot and intracellular cytokine staining. This finding is consistent with the ability of some gonococcal antigens to elicit Th1 type CD4 responses. In that study, cultured PBMC produced IFNγ, TNF-α, IL-4, and IL-10 in response to IgA protease antigen while only producing IFNγ and TNF-α in response to tetanus toxoid (39). This data suggests that the commensal bacteria colonization that resulted in cross reactive immune response to *N. gonorrhoeae* IgA protease elicited a polyfunctional immune response different in character from that induced by tetanus toxoid vaccination. In another *ex vivo* study of PBMC response to *N. gonorrhoeae*, cultured PBMC from 2 anonymous donors (and unknown *N. gonorrhoeae* infection history) were exposed to *N. gonorrhoeae* and CD4+ T cells were found to upregulate CD25, a T cell surface marker that is elevated in T regulatory cells and in activated CD4+ T cells. Further culture supernatant from these *N. gonorrhoeae*-treated PBMC was found to contain cytokines associated with Th1 (IFNγ), Th2 (IL-4), and Treg (IL-10) responses as well as the chemotactic factors IL-8 and MCP (42). Though the cellular source of these cytokines is unknown, the findings are consistent with the observation that *N. gonorrhoeae* infection induces a pleotropic immunologic response at the site of infection.

The role of cytotoxic T lymphocytes (CD8+ T cells), as well as cytotoxic innate immune cells like Natural Killer cells (NK cells), in gonococcal infection are unknown. Gonococcal PorB has been shown to induce robust IL-4 production in CD8+ T cells from *N. gonorrhoeae* infected individuals (38). However, contradicting effects of *N. gonorrhoeae* infection on CD8+ T cell function in humans are reported. In a longitudinal study of female commercial sex workers, Kaul and colleagues found that CD8+ T cell functional responses to HIV (in HIV infected subjects) and CMV (in HIV infected and uninfected individuals) were reduced during episodes of incident *N. gonorrhoeae* infection when compared to lymphocytes obtained when subjects were not *N. gonorrhoeae* infected (48). In contrast, a longitudinal study of HIV acquisition in a cohort of HIV negative female commercial sex workers demonstrated that early HIV-directed CD8+ T cell responses were more robust in subjects who were infected with *N. gonorrhoeae* at the time of HIV acquisition than in *N. gonorrhoeae* uninfected individuals (49). Additionally, asymptomatic anorectal *N. gonorrhoeae* infection in men who have sex with men taking pre-exposure prophylaxis to prevent HIV was found to be associated with increased activation marker in circulating CD8+ T cells (50). The adaptive immune response in cytotoxic T cell populations to *N. gonorrhoeae* infection remains largely unexplored at this time.

Cytokines from the IL-17 family are important for the recruitment of neutrophils and to induce localized antimicrobial

responses. Studies in a mouse model of *N. gonorrhoeae* infections suggest that the magnitude of Th17 responses to infection are more robust than Th1/Th2 responses (51). These findings are consistent with the neutrophilic inflammatory response commonly associated with *N. gonorrhoeae* infection in humans and the inverse relationship between systemic IL-17 and IFNγ levels reported in *N. gonorrhoeae* infected individuals (45). The relative resistance of *N. gonorrhoeae* to neutrophil mediated killing may render this Th17-skewed immune response less effective at clearing the pathogen than a more robust Th1/Th2-supported immune response might, (52, 53). Further, the ability of *N. gonorrhoeae* to induce IL-10 secretion and promote or stimulate Treg cells may also reduce humoral and cellular immune responses to infection (54). Supportive of this hypothesis, manipulation of the mouse immune system to block the induction of Treg related activity or to drive more robust Th1/Th2 responses leads to enhanced clearance of *N. gonorrhoeae* in model infection. Further, stimulation of Th1 responses through intravaginal IL-12 administration during infection in the mouse vaginal infection model resulted in enhanced clearance upon rechallenge when compared to mice initially infected without supplemental IL-12 (55). Because studies of lymphocyte proliferative response demonstrated that prior natural infection was associated with significant increase in proliferative response in subjects with current *N. gonorrhoeae* infection when compared to subjects without previous infection, it appears the cell mediated immunity being measured was inadequate to provide robust protection from repeat infection. Unfortunately, there are no studies to date that measure cellular immune responses to *N. gonorrhoeae* either in naïve or infected individuals in which the subjects were subsequently prospectively followed to determine whether these cellular immune responses correlate with any degree of protection from re-infection.

Despite public health efforts, gonorrhea still remains one of the most common sexually transmitted bacterial infections responsible for ∼100 million infections per year (56). The development of a gonococcal vaccine had been a focus of the field but no vaccine has been developed to the point of human trials since the failure of the *N. gonorrhoeae* pilin-based vaccine in clinical trials. The failure of the pilin vaccine was unexpected after it had shown success in preventing infection in a homologous strain challenge in humans and highlights the complex relationship between this human pathogen and its host (57). This review of published literature of human immunologic responses to *N. gonorrhoeae* largely supports the widely accepted supposition that *N. gonorrhoeae* manages to infect and re-infect humans using some combination of immunologic evasion and resistance to host mediators of clearance. However, there are some reports that support the possibility that natural clearance or protection from infection may develop in response to gonococcal infection in a small portion of individuals. The lack of a protective immune response observed in most infected humans coupled with the complex nature of antigen specific responses and limited animal infection models have all confounded past vaccine development efforts and stand in stark contrast to successful development of *N. meningitidis* vaccines. Recently, large scale efforts to develop effective vaccines toward serogroup B *N. meningitidis* which produces a poorly immunogenic capsule have led to the development of Outer membrane vesicle (OMV)-based vaccines which are effective at raising bactericidal antibodies against group B *N. meningitidis* in humans (58). Data emerging from Sexually Transmitted Infection surveillance in countries deploying OMV-based vaccine in mass vaccination campaigns suggest that the vaccine may induce some cross-species protection against *N. gonorrhoeae* infections (59–61). The mechanism by which this vaccine may be protective against *N. gonorrhoeae* is not fully understood. Further studies that evaluate the cellular and humoral responses directed against *N. gonorrhoeae* that develop after immunization with *N. meningitidis* OMV as well as comprehensive studies that combine sophisticated immunologic analysis and prospective monitoring of subjects are needed to understand both natural and the possibility of vaccine mediated immunity to *N. gonorrhoeae* infection.

AUTHOR CONTRIBUTIONS

AL and JD conceived and outlined this literature review and subsequently independently conducted literature searches. AL drafted initial manuscript, figures, and tables. JD edited and finalized the manuscript, figures, and tables.

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REVIEW
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Pathogenesis of *Neisseria gonorrhoeae* and the Host Defense in Ascending Infections of Human Fallopian Tube

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Neisseria gonorrhoeae is an obligate human pathogen that causes mucosal surface infections of male and female reproductive tracts, pharynx, rectum, and conjunctiva. Asymptomatic or unnoticed infections in the lower reproductive tract of women can lead to serious, long-term consequences if these infections ascend into the fallopian tube. The damage caused by gonococcal infection and the subsequent inflammatory response produce the condition known as pelvic inflammatory disease (PID). Infection can lead to tubal scarring, occlusion of the oviduct, and loss of critical ciliated cells. Consequences of the damage sustained on the fallopian tube epithelium include increased risk of ectopic pregnancy and tubal-factor infertility. Additionally, the resolution of infection can produce new adhesions between internal tissues, which can tear and reform, producing chronic pelvic pain. As a bacterium adapted to life in a human host, the gonococcus presents a challenge to the development of model systems for probing host-microbe interactions. Advances in small-animal models have yielded previously unattainable data on systemic immune responses, but the specificity of *N. gonorrhoeae* for many known (and unknown) host targets remains a constant hurdle. Infections of human volunteers are possible, though they present ethical and logistical challenges, and are necessarily limited to males due to the risk of severe complications in women. It is routine, however, that normal, healthy fallopian tubes are removed in the course of different gynecological surgeries (namely hysterectomy), making the very tissue most consequentially damaged during ascending gonococcal infection available for laboratory research. The study of fallopian tube organ cultures has allowed the opportunity to observe gonococcal biology and immune responses in a complex, multi-layered tissue from a natural host. Forty-five years since the first published example of human fallopian tube being infected *ex vivo* with *N. gonorrhoeae*, we review what modeling infections in human tissue explants has taught us about the gonococcus, what we have learned about the defenses mounted by the human host in the upper female reproductive tract, what other fields have taught us about ciliated and non-ciliated cell development, and ultimately offer suggestions regarding the next generation of model systems to help expand our ability to study gonococcal pathogenesis.

Keywords: *Neisseria gonorrhoeae*, fallopian tube, oviduct, organ culture, tissue explant, peptidoglycan, cilia, pelvic inflammatory disease

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INTRODUCTION

Infections with *Neisseria gonorrhoeae* (gonococcus, GC) most commonly begin at the cervix in females, which marks the dividing line between the lower reproductive tract (vagina, ectocervix) and the upper reproductive tract (uterus, fallopian tubes, ovaries, and endometrium). Cervical infections can be symptomatic or asymptomatic, but without treatment 10–20% of cervical infections ascend to cause infection of the upper female reproductive tract, including the endometrium and fallopian tubes (1). While ascending infection of the fallopian tube may be a dead-end for gonococcal transmission, it is a particularly consequential outcome for the unfortunate host. Fallopian tube infection leads to inflammation (salpingitis) and pelvic inflammatory disease (PID). Following PID, a woman's risk for ectopic pregnancy increases to 9% (from <2%), tubal-factor infertility increases to 16% (from <3%) (2), and chronic pelvic pain is experienced by 36% of patients (3). While the proportion of PID cases that are attributable to *N. gonorrhoeae* (<40%) has fallen relative to *Chlamydia trachomatis* (∼60%), gonococcal PID typically presents with more severe symptoms (4). The sharp rise in antibiotic-resistant gonococci raises the risk of reversing gains in preventing gonococcal PID (5).

Unlike many commonly studied bacterial pathogens, *N. gonorrhoeae* is not readily adaptable to laboratory animal models due to its exquisite adaptation to the human host. A female mouse model was developed nearly 20 years ago (6). With refinement in the intervening time, this model has proven very useful, especially in the understanding of complex systemic immune responses model reviewed here (7). Estradiol-treated mice become colonized following intravaginal inoculation and GC can ascend at least as far into the upper reproductive tract as the uterus (8). However, colonization is maintained for only about 10 days and resumption of the murine estrous cycle clears infection (7). Bypassing the vagina via transcervical inoculation allows for transient colonization of the uterus, with successful infection of the majority of animals for up to 24 h. The majority of animals then clear infection by 48 h (9). Despite the success of mouse models, there exist numerous biochemical, physiological, and morphological differences between murine and human female reproductive tracts, as well as between mouse and human immune systems. GC has evolved to exploit human versions of proteins for epithelial cell binding, iron acquisition, and immune evasion, among other features. For modeling human infections, a faithful reproduction of human disease occurs in experimental infection of chimpanzees (10, 11). Studies also can be performed on the infection of human male volunteers. However, both of these models are expensive and not practical for large-scale use. Moreover, human experimental infection necessarily excludes the use of females due to the risk of severe complications. Though the male urethral infection model continues to provide many important insights into host and pathogen biology, this review will focus primarily on modeling infection of the human upper female reproductive tract as the male model has been reviewed elsewhere (12, 13).

As an alternative to animal models for understanding ascending infections and the development of PID, portions of human oviducts (fallopian tubes) can be maintained in culture for days to weeks (14). While pre-menopausal samples are the best for assuring vigorous ciliary activity (15), the hormonal status of donors has no noticeable effect on ciliary activity (16). Therefore, samples obtained from any stage of the menstrual cycle are suitable for use in organ culture. Explants provide an opportunity to study gonococcal infections on a human female epithelial surface that is targeted during natural infection, complete with the complex mixture of ciliated and secretory epithelial cells and multi-layered tissue architecture. This review is intended to summarize what we have learned from fallopian tube organ culture infections with gonococci, what is known about the immunological capabilities of the fallopian tube, and how this immunology relates to our understanding of gonococcal host-pathogen interactions. Lastly, we address how the improvement of human organ and organ-like models are expanding our ability to probe specific molecular and genetic interactions between *N. gonorrhoeae* and the human host.

INVADING THE FALLOPIAN TUBE

Defining a Tissue Explant Infection Model for *N. gonorrhoeae*

Prior to the use of human fallopian tube organ cultures as a model ciliated epithelium, tracheal cultures had been used to study infectious agents. Trachea were shown to survive *ex vivo*, displaying ciliary activity for 3–5 weeks (17). In the first published attempt to model gonococcal interactions with ciliated epithelia, fallopian tubes were used alongside tracheal samples from embryonic chicken, cow, human, and adult mouse (16). Non-piliated gonococci were found to grow well on fallopian tube tissue, with an inoculum as low as 10 colony forming units (CFU) reaching a maximal density (10^7-10^{10} CFU/mL) in 2–4 days. A higher inoculum (10^3 CFU) reached a maximum density after 1–2 days and caused a complete loss of ciliary activity by 5–6 days. For comparison, uninfected fallopian tube tissue maintained beating cilia for between 14 days and 1 month (or more). Tissue culture media such as Eagle's Minimal Essential Medium (MEM) is sufficient to support tissue survival, though gonococci grow poorly in this media (15). Gonococci will grow, however, in conditioned media from fallopian tube or media supplemented with tissue homogenate. Work that compared rabbit oviducts in culture to human fallopian tube revealed that, similar to the previous work in trachea, the presence of any tissue supports the multiplication of GC in culture. Gonococci, however, neither bind to nor damage rabbit oviduct, and do not cause any reduction in ciliated cell activity (18). Piliated gonococci likewise adhered poorly to the mucosal epithelium of rabbit, pig, cow (19), and guinea pig (20). In all cases, no noticeable histopathology or loss of ciliary activity was observed in animal oviducts compared to human fallopian tubes infected in parallel. One of the seminal observations, made with both piliated and non-piliated strains, is that gonococci bind preferentially to secretory (non-ciliated) cells, though it is the ciliated cells that later die (18, 21). Gonococci are also able to quickly invade the apical side of non-ciliated cells

(within ∼20 min) (18), and then exit from the basolateral side (transcytosis) but are not observed invading or residing inside ciliated cells (21). When non-pathogenic *Neisseria pharyngis* (now known as *N. cinerea*) was grown in fallopian tube organ culture, the bacteria survived at similar levels to *N. gonorrhoeae*, but failed to elicit any of the decrease in ciliary activity that is characteristic of GC (15), suggesting that human pathogenic *Neisseria* possesses unique factors capable of damaging human female reproductive tract epithelia.

Epithelial Damage and the Inflammatory Response to Gonococci

Early work in the human fallopian tube organ culture model established that GC could colonize explants and recapitulate the damage seen in patients with gonorrhea (22). The specific damage of primary concern is the death of ciliated epithelial cells, which have physiologic functions important in fertility. Loss of the ability of ciliated cells to participate in transporting the fertilized ovum to the uterus is considered a major predisposing factor for tubal factor infertility and ectopic pregnancy (23). In culture, uninfected fallopian tube explants display robust ciliated activity for more than 2 weeks, but tissues infected with non-piliated gonococci show decreases in ciliary activity starting at about 36 h post-infection and exfoliation of ciliated cells starting around 64 h (18). Complete loss of ciliated cell activity occurs by 4–6 days post-infection (16, 18). Multiple groups observed that a decrease in ciliary beat frequency precedes the appearance of visible damage of the epithelial surface (24, 25). The magnitude of reduction in ciliary activity and rapidity of sloughing is more pronounced in infections with piliated gonococci compared to non-piliated, even with equal or fewer CFU recovered from tissues (25).

Ciliated cells are the first to die despite the attachment of gonococci to non-ciliated cells. This observation led to a search for soluble factors (such as toxins) present in filter-sterilized gonococcal broth cultures (cell-free supernatant) and filter-sterilized media from gonococci-infected fallopian tube cultures. Both supernatants were found to contain a heat-stabile, toxic component that is partially removed by adsorption to limulus amoebocyte lysate (LAL), and capable of causing the same ciliated cell death as observed in gonococcal infections (26). This toxic effect was attributed to lipopolysaccharide (LPS), which is present in gonococci as a low-molecular weight version lacking the repeating O-antigen and referred to as lipooligosaccharide (LOS). LOS was purified and shown to decrease ciliary activity at lower concentrations than the microgram quantities measured in infected organ cultures (27). The toxic effect of gonococcal LOS, like damage from gonococcal infection, also appeared to be a human-specific phenomenon, not affecting the ciliary activity of rabbit, pig, or cow oviducts (28). This observation would seem curious now, as LPS/LOS signaling via the eukaryotic immune sensor Toll-like receptor 4 (TLR4) has been characterized as a conserved pathway leading to inflammatory responses in all of the above species (29, 30). Adsorption of LOS with LAL, however, did not eliminate all of the soluble toxic activity produced by gonococci, implicating another factor(s) causing toxicity. Around the same time, it was just beginning to be recognized that gonococci release soluble fragments of peptidoglycan (PG), in particular monomeric fragments consisting of *N*-acetylmuramic acid and *N*-acetylglucosamine sugars linked to three- or four-amino acid long peptide chains (with 80% as tripeptide, 20% as tetrapeptide) (31, 32). Treatment of organ cultures with isolated PG monomers, reduces ciliary activity and causes death and sloughing of ciliated cells (33). The later isolation from *Bordetella pertussis* of tracheal cytotoxin (TCT), a tetrapeptide PG monomer identical to the minority monomer species released by gonococci, reinforced the idea that small PG monomers can cause ciliated cell cytotoxicity, in this case in the trachea of hamsters (34, 35). The further discovery that PG is found as a contaminant in crudely purified LPS preparations (36), and the recognition that PG monomers are sensed differently by the same receptor in different species (37), implicates PG as a major mediator of species-specific oviduct toxicity. Certainly, both LOS and PG are toxic products, abundantly released from growing gonococci and capable of contributing individually to inflammation. Together, it is likely they have an even greater impact, as PG monomers are known to synergize with LPS to provoke a larger host response than either alone (38).

While gonococcal LOS and PG fragments are ascribed causative roles in epithelial damage, neither is a "toxin" in the same sense as a lytic pore-forming toxin or a compound capable of poisoning cellular processes to create a direct toxic effect. Rather, LOS and PG are recognized by the host as pathogen-associated molecular patterns (PAMPs) and induce programmed defense responses. One of the first host inflammatory responses measured from gonococcal infection of fallopian tube organ cultures was the induction of tumor necrosis factor (TNF) (39). Addition of TNFα to fallopian tube explants reduces ciliary activity in a dose-dependent manner, and reproduces the characteristic death and sloughing of ciliated cells (**Figure 1**). Rising concentrations of TNFα during gonococcal infection correlate linearly with a decrease in ciliary activity (40). In the analogous *B. pertussis* system, it should be noted that interleukin (IL) 1 (and not TNFα) was implicated as the host factor driving respiratory epithelial damage. IL-1 addition was shown to be capable of inducing ciliated cell death similar to that seen in TCT treatment of hamster tracheal rings (41). Though the host organism and infection system are different, the role of IL-1 is noteworthy as this cytokine is also implicated as the driver of epithelial damage in *Chlamydia trachomatis* infections of human fallopian tube organ cultures (42).

Gonococcal infection is capable of inducing transcription of numerous cytokines and chemokines in epithelial cell lines such as ME-180, HeLa, and HaCaT via activation of the transcription factor nuclear factor kappa-B (NF-κB) (43). In fallopian tube explants, TNFα and IL-1β, as well as the inflammatory cytokines/chemokines IL-6, monocyte chemoattractant protein-1 (MCP-1, CCL2), macrophage inflammatory protein-1β (MIP-1β,CCL4), and granulocyte-macrophage colony-stimulating factor (GM-CSF) are made in response to gonococcal infection and detected by enzyme-linked immunosorbent assay (ELISA) after as little as 3 h (44, 45). While levels of TNFα, IL-1β, IL-6, and MCP-1 seem to generally

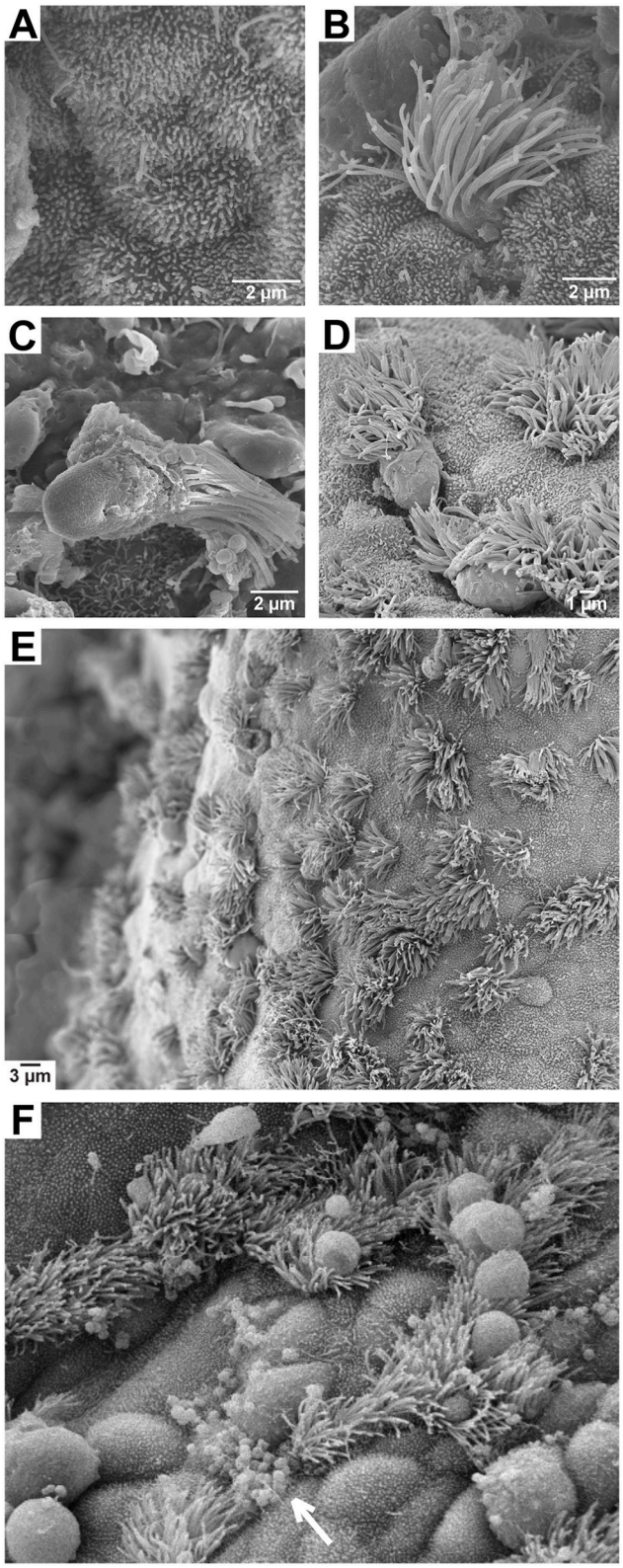

FIGURE 1 | Scanning electron micrographs of fallopian tube explants. **(A)** Non-ciliated (secretory) epithelial cell; **(B)** ciliated epithelial cell; **(C)** ciliated

(Continued)

FIGURE 1 | epithelial cell sloughed during gonococcal infection; **(D)** ciliated cells sloughing following a 24 h treatment with 2 ng/mL TNFα; **(E)** an untreated epithelial layer displaying normal cell morphology; **(F)** an epithelial layer during gonococcal infection showing binding of most bacteria to non-ciliated cell surfaces (arrow) and swelling of cells that precedes ciliated cell sloughing.

increase out to 24 h, MIP-1β and GM-CSF appear to peak at 12 h and decline by 24 h. The kinetics of some cytokine responses were shown to vary between piliated and non-piliated variants, with cytokine induction by piliated gonococci potentially delayed by several hours (45). Since the vast majority of gonococci recovered from symptomatic natural infections are piliated (46, 47), a delay in cytokine induction in fallopian tube explants may indicate a role for pili in suppressing certain immune responses during ascending infection.

Nitric oxide (NO) is another component of the inflammatory host response that is produced on mucosal epithelia, in particular NO made by inducible nitric oxide synthase (iNOS, encoded by *nos2*). In hamster tracheal epithelial (HTE) cells, TCT and IL-1 were shown to induce nitrite production (a proxy for NO), and applying nitric oxide synthase inhibitors prevented the loss of ciliary activity in hamster tracheal rings treated with TCT (48). In the hamster trachea, NO production localizes only to the non-ciliated (secretory) epithelial cells (49). Though intriguing, this finding regarding the distribution of NO production has not been reproduced in humans or in reproductive tract tissues. During gonococcal infection of fallopian tube organ cultures, iNOS transcription is induced ∼35-fold, but treatment of uninfected fallopian tube tissues with the NO donor *S*-Nitroso-*N*-acetylpenicillamine (SNAP) did not induce cellular damage as measured by lactate dehydrogenase (LDH) release (50). Treatment of infected tissues with iNOS inhibitors also produced mixed results in reducing cellular damage (50). The inhibition of fallopian tube damage that was seen during treatment with N(G)-Nitro-L-arginine methyl ester (L-NAME) corresponded with a reduction in bacterial CFU. A decrease in bacterial proliferation presumably accounted for a corresponding reduction in tissue damage. Interestingly, a reduction in viable gonococci during inhibition of iNOS aligns with data that iNOS activity increases gonococcal survival within primary human cervical epithelial (pex) cells (51). While it is unclear whether NO participates directly in the damage observed in the fallopian tube during gonococcal infections, GC has adapted methods to avoid killing and perhaps even exploit NO during host colonization.

Matrix metalloproteinases (MMPs) are host enzymes that function in the degradation of extracellular matrix, disassembly of cellular junctions (including for immune cell infiltration), and activation (by cleavage) of cytokines and growth factors (52). Numerous bacteria or bacterial products (including PG and LPS) have been shown to induce various MMPs (including MMP1, 2, 3, 7, 9, 10, and 11) (53–56). When bacteria induce MMPs, host tissue can sustain collateral damage, such as when Gram-positive *Enterococcus faecalis* activates MMP9, contributing to adverse outcomes of surgical wound infections (57). During gonococcal infection of fallopian tube epithelial cells, MMP2 accumulates intracellularly while MMP9 secretion is increased

(MMP3 and MMP8 levels are unchanged) (58). In fallopian tube explants, MMP8 expression is increased following GC infection, while transcription of MMP3 and MMP9 (as well as the TIMP metallopeptidase inhibitor 1, TIMP-1) are unchanged (59). The functions and localization of MMPs, when coupled with data indicating their induction during fallopian tube infection, makes these enzymes prime candidates for mechanistic involvement in epithelial damage. In cell culture, gonococci cause disruption of E-cadherin and β-catenin from adherens junctions between fallopian tube epithelial cells (60). GC also disrupt the apical junctions of polarized HEC-1-B (endometrial) and T84 (colonic epithelial) cells by activating epidermal growth factor receptor (EGFR) signaling (which also involves redistribution of β-catenin) (61). The disassembly of apical junctions is also seen in an endocervical tissue model of infection, where GC activates intracellular non-muscle myosin II and induces calcium flux to promote the exfoliation of columnar epithelial cells (62). Additional investigation is needed into the composition of cellular junctions between ciliated and non-ciliated fallopian tube epithelial cells, the activity of bacterially-induced MMPs, and the intracellular signaling pathways activated by gonococci to determine what factors make ciliated cells especially sensitive to exfoliation.

The exact mechanisms by which ciliated cells die and are removed from the epithelial layer remain unknown. Morphologically, cells do not appear to lyse as would be expected from necrosis or pyroptosis (though IL-1β is released during infections). In cultured human fallopian tube epithelial cells, GC inoculated at various multiplicities of infection (MOI = 1, 10, or 100) all result in production of TNFα, which can cause apoptosis. However, only the lowest MOI showed significant Caspase-3 activation (indicative of apoptotic cell death) (63). Since TNFα addition alone can induce apoptosis and addition of more GC (higher MOI) blocks the TNFα-dependent apoptosis, it can be hypothesized that GC contact or some GC-produced soluble factor produces an anti-apoptotic effect. Indeed, GC is known to increase the expression of anti-apoptotic factors in human urethral epithelial cells (64, 65), and human End1 endocervical epithelial cells (66). GC also confers protection from apoptosis in neutrophils (67, 68). However, GC also induces pro-apoptotic genes in HeLa cells (69). The outer membrane porin PorB has been implicated in induction of apoptosis by (1) trafficking to mitochondria in murine bone marrow-derived macrophages (BMDMs) and human THP-1 macrophages, and (2) initiating the intrinsic pathway of apoptosis in mouse BMDMs (70). During fallopian tube explant infections, GC were more often seen contacting (or in close proximity to) non-ciliated cells, which could facilitate the induction of a protective response. Ultimately, a better understanding is needed of the differences between ciliated and non-ciliated cells in their ability to sense and respond to TNFα and other components of the host defense response.

Factors Conferring Adherence to Fallopian Tube Epithelia

Adhering to host surfaces, whether to facilitate physical anchoring for replication or to induce uptake into host cells, is a critical step in the life cycle of many bacterial pathogens. Such is the case for GC, which can multiply extracellularly on mucosal surfaces, or proceed through a stepwise process of first binding tightly to columnar epithelial cells, being endocytosed, transiting through epithelial cells, and exiting into the subepithelial space (71, 72). Early observations of *N. gonorrhoeae* adherence during human fallopian tube organ culture infection focused on the two best known (and most easily observable) mediators of adherence: pili and opacity (Opa) proteins. Piliated GC bind to (and damage) the mucosal surface more rapidly than non-piliated variants of the same strain (25). Piliated gonococci were also observed binding to the tips and surfaces of microvilli on non-ciliated cells (73), a phenomenon later observed in infections of cultured HEC-1-B endometrial carcinoma cells as a precursor to cellular invasion (74). The exact receptor that gonococcal pili engage during fallopian tube infection for either adherence or invasion is unknown. Both CR3 (a CD11b/CD18 integrin heterodimer) and CD46 (MCP) have been implicated as ligands for pili (75, 76), and both are found on the fallopian tube epithelium (77, 78). Expression of CR3, however, decreases progressively in ascending tissues, reaching its lowest relative level in the fallopian tube (77). In addition, questions remain as to whether the interaction of pili with CD46 functions to mediate adherence or rather a downstream intracellular signaling event (79–82). In the endometrium, carcinoembryonic antigen-related cell adhesion molecules (CEACAMs/CD66), asialoglycoprotein receptor (ASGP-R), and CR3 have each been implicated in gonococcal adherence (83).

Gonococci can still bind to and damage fallopian tube epithelia through pilus-independent mechanisms (18), and Opa proteins alone are sufficient to confer adherence to fallopian tube epithelium (84). In the only study to look exclusively at the role of Opa proteins in attachment to fallopian tube, Opa- variants of F62-SF were found to bind exclusively to non-ciliated cells (as observed previously) as did OpaC+ and OpaD+ strains (which bound to microvilli). Strains expressing OpaB, however, were seen on both ciliated and non-ciliated cells, with OpaA-expressing strains binding to ciliated cells in large clumps (85). Interestingly, the OpaC and OpaD-expressing variants were judged the most damaging to epithelia while OpaA-expressing were the least damaging. Despite Opa proteins being well characterized as adhesins and routinely associated with successful human infections (86), early work in fallopian tube organ culture suggested that piliated and transparent (Opa-) bacteria bind better (by 10–100x) to fallopian tube explants than piliated and opaque (Opa+) bacteria (22, 73). In laparoscopic sampling from the fallopian tubes of women with salpingitis, the same group recovered predominantly GC with transparent colony phenotypes (>95% Opa-) while endocervical isolates were 50% or greater Opa+ (87). The majority of Opa proteins (which vary in number between gonococcal strains) bind to distinct CEACAMs, though at least one Opa binds instead to heparin-sulfate proteoglycans (HSPG) (88). In a study of low-passage isolates of GC from cervix and male urethra, the majority of strains bound to transfected Lec11 cells (a CHO derivative) stably expressing CEACAM1, CEACAM5, or CEACAM6. Fewer showed robust binding to CEACAM3 and very few bound

CEACAM8 (89). Human CEACAM5, when expressed in mice, also contributes to increased recovery of GC during murine lower genital tract infection (90). The expression levels of particular CEACAMs on cells of the human female genital tract has been questioned, however, with evidence that CEACAM1, 3, 5 (CEA), 6, and 8 are not highly expressed on primary fallopian tube, primary cervix, HEC-1-B endometrial cells, or HeLa cervical carcinoma cells (91). It is not known which CEACAM (or HSPG) is the most available target of Opa proteins in the fallopian tube, nor is it known what threshold level of expression is needed for Opa-CEACAM binding in any given tissue. It is clear that Opa proteins facilitate adherence to cultured epithelial cells and contribute to bacterial fitness during successful colonization of the murine lower genital tract (92). In primary human ectocervical and endocervical cells, however, the absence of Opa expression does not decrease adherence (76). It is still possible that Opa proteins contribute to the mix of adhesins needed to address the changing receptor availability and immunological context encountered during the progression of ascending from the endocervix to the fallopian tube (83). In a study of seven patients with acute salpingitis (fallopian tube inflammation), laparoscopic isolation of bacteria from fallopian tube and cul-du-sac revealed a higher proportion of Opa- than Opa+ in these tissues (22). The laparoscopy samples also had a consistently higher proportion of Opa- GC than matched cervical isolates from the same patient. These data suggest that whatever factors are driving Opa protein production at the endocervix, Opa proteins may be turned off or selected against when bacteria are infecting the fallopian tube.

Gonococcal lipooligosaccharide has already been discussed as an important trigger of host inflammatory responses, but LOS on the cell surface may also function in bacterial binding and invasion of fallopian tube epithelial cells. Several different gonococcal LOS variants bind to Galectin-3, a β-galactoside-binding protein expressed on non-ciliated cells of fallopian tube epithelium as well as on HEC-1-B endometrial adenocarcinoma and PC3 human prostate adenocarcinoma cells (93). The "triggering receptor expressed on myeloid cell-2" (TREM-2) was also identified as a ligand for gonococcal LOS, and is constitutively expressed on human fallopian tube epithelium, ME-180 and HeLa cervical carcinoma cells, ectocervical Ect1/E6E7 cells, endocervical End1/E6E7 cells, vaginal Vk2/E6E7 cells, and THP-1 monocytic cells (94). The resident microbiota in the female lower reproductive tract contribute sialidase activity that processes the terminal sialylation present on LOS, a process that has been implicated in promoting transmission to males by unmasking a terminal galactose that binds to ASGP-R on urethral epithelial cells (95, 96). It is unclear how the sialylation state of LOS impacts the pathogenesis of fallopian tube infection. Increasing LOS sialylation is known to decrease Opa-dependent invasion of Chang conjunctival and ME-180 cervical epithelial cells (97). Several studies also report rapid internalization of gonococci into fallopian tube secretory epithelial cells (21, 22), perhaps implicating a low level of LOS sialylation during ascending infection.

Natural hormonal cycling likely plays a role in the expression of gonococcal factors important for adhesion, immune evasion and virulence. Before specific Opa proteins were recognized as the factors determining colony opacity/transparency, variations in the visual appearance of isolates were noticed to change (along with the likelihood of recovering viable gonococci) based on the stage of the menstrual cycle (47). Isolates obtained in close temporal proximity to menstruation were found to lack the surface proteins associated with opacity. The overall variation in recovery phenotypes during the menstrual cycle was attributed to the action of progesterone (or substances acting similarly to progesterone). While progesterone has been investigated for its ability to enhance survival of gonococci in primary cervical epithelial (pex) cells (51), its role in ascending infection is unknown. However, since progesterone causes a significant lowering of ciliary beat frequency in fallopian tube (98), progesterone could reasonably be expected to have some impact on gonococcal adherence or colonization in this tissue. Additionally, fallopian tube tissue from users of both hormonal and non-hormonal (copper intrauterine device) contraception display changes in the surface expression of CD46 and the HSPG syndecan-1, but not CD66 (CEACAM) (78). Much remains to be determined about how bacteria, adherent at the cervix, transit the uterus to enter the fallopian tube. It is likely that gonococci have adapted to utilize numerous host receptors in sequence (83), and are able to adjust accordingly to whatever tissue environment they find themselves in as they make their way into the upper female reproductive tract.

Insights From *Chlamydia Trachomatis* Infections in Fallopian Tube Explants

Chlamydia trachomatis (Ct) is the most commonly reported sexually transmitted infection (STI) and another bacterium that can ascend to the fallopian tubes, causing PID and tubal-factor infertility. Fallopian tube organ culture explants have also been used to study the pathogenesis of Ct, with early observations in this system noting that Ct is able to bind to and infect both ciliated and non-ciliated cells (99). Unlike GC infection, ciliated cell function did not appear to be affected by Ct, though microvilli were lost on infected non-ciliated cells, which became rounded and lost attachment to neighboring cells. By 72 h post-infection, ruptured cells could be observed, characteristic of the cell lysis that results from release of infectious elementary bodies (EBs) at the end of the Ct intracellular life cycle. The observed loss of cellular polarity and disruption of cell-cell junctions in Ct-infected fallopian tube is accompanied by β-catenin recruitment from adherens junctions to Chlamydial inclusions (100). While disassembly of junctions likely contributes directly to epithelial disruption, β-catenin is also tightly regulated at the cellular level as a component of the developmentally important Wnt signaling pathway. Increased Wnt signaling during Ct infection induces numerous changes in fallopian tube tissue, including up-regulation of the stem-cell marker Olfactomedin 4 (OLFM4) (100), which is a target of NF-κB and Notch signaling pathways. OLFM4 also participates in the curtailing of innate immune responses by negatively regulating nucleotide-binding oligomerization domain-containing protein 1 (NOD1)- and NOD2-dependent NF-κB activation (101).

Cultured epithelial cell lines (HeLa, SiHa, HT-29, SW620, and primary endocervical) infected with Ct secrete proinflammatory cytokines (IL-8, IL-6, GM-CSF, and GROα/CXCL1), but cytokine secretion is delayed until 20–24 h post-infection, after lysing cells release IL-1α (102). Blocking IL-1α blunts the cytokine burst, suggesting that neighboring epithelial cells are sensing IL-1α and initiating an amplification of the inflammatory response. In fallopian tube organ cultures, addition of exogenous IL-1α was shown by scanning electron microscopy (SEM) and histological staining to cause damage similar to Ct infection, with IL-1 receptor antagonist (IL-1RA) and p38 mitogen-activated protein (MAP)-kinase blockade (downstream of IL-1 sensing) reducing epithelial damage (42). The ability of cytokines (TNFα in the case of GC, IL-1α for Ct) to exacerbate immune-driven pathology is common to both infections, as is potentially the role of matrix metalloproteinases (MMPs) in tissue disruption. Transcriptional analysis of Ct infecting monolayers of HEp-2 cells revealed induction of MMP2 and MMP9 (103), the same MMPs with altered expression during GC infection of fallopian tube (58). Though Ct and GC have different lifestyles that bring them into conflict with the host in different ways, both organisms induce a pro-inflammatory response in the fallopian tube epithelium, where the long-term sequelae of infection appear to be the result of host-driven inflammatory pathology.

DEFENDING THE FALLOPIAN TUBE

Pattern Recognition Receptors in the Innate Immune Response

Colonization of GC in the upper female reproductive tract (FRT) involves bacteria binding to and interacting with epithelial cells. Epithelial cells are ready to respond to the presence of invading pathogens through the expression of a variety of microbial pattern recognition receptors (PRRs). Surveys of PRR expression throughout the FRT revealed expression of all identified toll-like receptors (TLR1-10), as well as cytosolic sensors NOD1, NOD2, retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) (104–107). In general, receptor expression appears to be constitutive and nearly ubiquitous across different regions of the FRT, but notable differences exist in the levels of receptor expression between tissues. TLR2 transcripts are higher in the fallopian tube and cervix than other FRT sites, while TLR4 transcript is higher in the fallopian tube and endometrium with lower expression in the lower tract (104). Immunohistochemical staining confirmed the presence of TLR1-3 and 5-6 throughout the FRT, and indicated detectable TLR4 only in the fallopian tubes, uterus, endometrium, and endocervix (TLR7-10 were not tested in this work) (105). The absence of TLR4 from the ectocervix and vagina would presumably reduce the triggering of inflammatory signaling that may be disadvantageous for maintaining a healthy vaginal commensal community. Transcript for TLR10 is perhaps the most restricted, having first been reported in the endometrium (108). That finding was disputed by later work that surveyed the entire FRT and found detectable expression only in the fallopian tube (106). Differences in detection may be due in part to variations in expression that occur at different points in the menstrual cycle reviewed in (109). A high relative expression of TLR10 in the fallopian tube is particularly interesting since TLR10 appears to act as an anti-inflammatory TLR. TLR10 heterodimerizes with TLR2 (which also has high expression in the fallopian tube) to inhibit inflammatory signaling and promote the production of anti-inflammatory IL-1 receptor antagonist (IL-1Ra) (110). The presence of TLR10 may then serve as a check on out-of-control inflammation in the Fallopian tube. The localization of TLRs can also be further restricted (or enriched) at the cellular level within the fallopian tube. Though TLR4 expression was reported to be generally high in fallopian tube, this receptor may be preferentially present on the surface of oviductal stromal fibroblasts, underneath the epithelial layer, and not on the epithelial cells themselves (111). The absence of TLR4 on epithelial cells could function to protect against the spurious activation of pathologic inflammation in the fallopian tube. However, the demonstrated ability of GC to disrupt epithelial integrity and invade through the epithelial layer (18, 21) likely puts the bacterium in proximity to TLR4 on fibroblasts. Some evidence also exists that ciliated and non-ciliated epithelial cells express TLRs at different levels, with ciliated cells expressing all 10 TLRs at higher levels than non-ciliated cells (112). It is unclear as of yet how differential responsiveness to TLR agonists might relate to the different cell fates seen during GC infections.

The cytosolic PRRs NOD1 and NOD2 recognize various fragments of PG (113), which are shed in abundance by gonococci and known to cause damage during fallopian tube infection (33). Synthetic muramyl peptides, some of which are similar to those naturally released by gonococci, can be endocytosed by HEK293T cells to activate NOD1 and NOD2 (114). When the fallopian tube, endometrium, endocervix, and ectocervix regions were surveyed for NOD receptor expression, all tissues were found to express NOD1, NOD2, and the adaptor receptor interacting serine/threonine kinase 2 (RIPK2) (106). Fallopian tube was shown to have the highest relative expression of both NOD1 and NOD2 compared to other FRT tissues (107). Primary fallopian tube epithelial cells were also shown to be capable of generating an IL-8/CXCL8 response to the addition of the NOD1 agonist D-gamma-Glu-*meso*-diaminopimelic acid (iE-DAP) and the NOD2 agonist muramyl dipeptide (MDP) (106). Fallopian tube organ cultures treated with conditioned media from *N. gonorrhoeae* or *N. meningitidis* broth cultures both induce an IL-8 response proportional to the release of human NOD1 agonist (115).

Gonococcal products, whether on the bacterial surface, secreted, or delivered by membrane vesicles, have been shown to interact with several TLRs and NOD receptors, which likely initiate the early innate immune signaling during ascending infection. Cell-free supernatant and gonococcal lysates (containing whole PG sacculi) activate NF-κB via human NOD1 and NOD2 (116). The NOD1 activation is dependent upon the periplasmic conversion of a gonococcal cell wall rich in tetrapeptide-stem PG into soluble fragments with primarily tripeptide stems that are agonists for human NOD1 (117). Gonococci can activate human NOD2 with released glycosidically-linked peptidoglycan dimers, and with multimeric

PG (as might result from cell lysis) that is converted by host lysozyme to produce reducing disaccharide-containing PG fragments (118). Gonococcal lipooligosaccharide activates TLR4 signaling, including both the MyD88 pathway that activates NF-κB and the interferon regulatory factor 3 (IRF3) pathway that activates Type I interferons (119). The acylation state of the lipid A molecule is critical to gonococcal TLR4 activation, with the wild-type hexa-acylated version required for TLR4-dependent signaling (120). The placement of various sialic acid moieties (sialylation) on LOS appears not to influence TLR4 activation (121), while the presence of phosphoryl substituents on the lipid A molecule does influence TLR4 activation (119). Pathogenic *Neisseria* are potent activators of TLR4 signaling in part because they are prone to membrane blebbing, or the release of outer membrane vesicles, which functions to spread LOS, membrane proteins and associated intracellular cargo out from the cell, increasing the potential for bacterial products to contact PRRs (122–124).

Despite the lack of TLR4 expression in certain regions of the FRT, and a confirmed lack of expression in isolated vaginal, ectocervical and endocervical cells lines, GC is still able to activate NF-κB and induce proinflammatory cytokines in End-1 endocervical cells in the absence of TLR4 (125). TLR2 has long been recognized as another PRR activated by pathogenic *Neisseria*, an activity that has been largely attributed to recognition of the major outer membrane porin (PorB) (126, 127). Most studies of porin-TLR2 interaction have involved PorB from *N. meningitidis,* which is considered a stronger TLR2 agonist than *N. lactamica* PorB, which has been investigated for its vaccine adjuvant potential (128, 129). Gonococcal PorB, however, has recently been shown to have low TLR2 agonist activity when protein is refolded during purification, and higher TLR2 agonist activity when PorB is unfolded or aggregated (130). The outer membrane lipoprotein, Lip (also known as H.8 antigen) from GC has been shown to activate an NF-κB reporter in HEK293 cells expressing TLR2, and induce IL-6 and IL-8 from End-1 endocervical epithelial cells (131), confirming that GC possesses multiple potential TLR2 agonists.

Gonococci are able to activate additional cellular surveillance pathways, in particular activation of TRAF-interacting protein with forkhead-associated domain (TIFA) (132), and cyclic-GMP-AMP synthase (cGAS) (133). TIFA-dependent signaling is initiated by the intracellular detection of heptose-1,7-bisphosphate (HBP), a byproduct of gonococcal LOS production. Though a role for HBP has not yet been explored in fallopian tube infections, gonococcal supernatant containing HBP induces inflammatory responses in cultured End-1 endocervical epithelial cells, human macrophages, and human neutrophils (132). HBP has been shown in *Helicobacter pylori* infections to produce an earlier NF-κB response than NOD1 agonist (134). The cGAS enzyme is activated by detection of cytosolic double-stranded DNA (dsDNA) and initiates STING/TBK-1/IRF3 activation leading to induction of type I interferon. During GC infection of human monocytes (THP-1 cells), TLR4 and cGAS synergize to induce production of interferon-β (IFN-β) (133). Even with this observation, it is uncertain what role IFN-α or IFN-β has during gonococcal infection, since IFN-β can be both beneficial and detrimental to the host in clearing infection. In addition, the source of cytosolic gonococcal dsDNA sensed by cGAS remains uncertain, though presumed to originate from spontaneous bacteriolysis. It is therefore plausible that the proliferation of extracellular bacteria releasing TLR4 agonist, while some bacteria are phagocytosed and lyse inside responding macrophages, creates a scenario for increased IFN-β production during fallopian tube infection.

Immune Cells in Fallopian Tube Surveillance

Like other mucosal surfaces, the fallopian tube contains a complement of resident immune cells that can be found within the epithelial layer and just behind the epithelium in the lamina propria. A flow cytometry-based survey of the entire human female reproductive tract across 28 patients ranging from 26 to 66 years old revealed 6–20% of the total cellular content to be leukocytes ($CD45^+$), with fallopian tube and uterus containing a higher proportion of immune cells than the cervix or vagina (135). Across all tissues, 30–60% of the leukocytes were T cells ($CD3^+$), with granulocytes ($CD66b^+$, likely polymorphonuclear leukocytes, PMNs) as the major population observed in the fallopian tube. However, a later study that focused exclusively on the fallopian tube epithelium and utilized both flow-cytometry and immunohistochemistry with an updated and expanded set of cellular markers reached a somewhat different conclusion on leukocyte distributions (136). Ardighieri et al. (136) surveyed 10 patients (5 pre-menopausal, 26-35; 5 post-menopausal, 63-73) and observed macrophages ($CD68^+$ $CD163^+$) and dendritic cells (DCs) ($CD11c^+$) as the most abundant innate immune cells present in the fallopian tube, at ratios (to total nucleated cells) of ∼1/30 and 1/21, respectively. While all combinations of $CD11c^{+/-}$ and $CD163^{+/-}$ cells (overlapping macrophage and DC markers) were present, $CD11c^+$ $CD163^-$ DCs were abundant. These cells were observed at regular intervals, with cell bodies in the basal lamina and long intraepithelial projections facing toward the lumen. $CD11c^+$ $CD163^+$ cells were the most abundant population in the lamina propria and muscle wall (under the epithelium). In this same analysis (136), neutrophils were noted as a minor population localized almost exclusively intravascularly and present only in the lamina propria and muscle wall, the same localization as minor populations of mast cells ($CD117^+$), plasmacytoid DCs ($CD303^+$), plasma cells ($CD138^+$), and regulatory T cells ($CD3^+Foxp3^+$). Whether or not neutrophils are a major population in the normal fallopian tube, these cells are well characterized as important components of the cellular response to GC infections. Neutrophils migrate rapidly from the bloodstream to sites of tissue inflammation and are found in high numbers in gonococcal infection of the male urethra and female cervix. As the role of neutrophils in GC infection of the female reproductive tract has recently been reviewed (137), the following section will focus on other fallopian tube-resident immune cell types and the outcomes of their potential interactions with gonococci.

Dendritic cells are critical front-line phagocytes and potent antigen-presenting cells, which function in the activation of T cells (138). DCs that encounter antigen in the presence of proinflammatory signals are induced to produce IL-12 and costimulatory molecules that encourage the development of T helper cells. Contact with GC, however, has been shown to induce expression of anti-inflammatory molecules (IL-10, PD-L1) from human and murine DCs, and both human and murine DCs exposed to GC are less able to stimulate proliferation of $CD4^+$ T cells (139, 140). Porin (PorB) delivered via outer membrane vesicles (OMVs), has been shown to contribute to the suppressive effect of GC on DCs, in spite of any ability of porin to engage stimulatory TLR2 signaling (130). Though the mechanics of GC suppression of DC activity are still incompletely understood, several potentially important receptor-ligand interactions have been identified. Gonococcal LOS binds to C-type lectin receptors on DCs, with variations in glycosylation influencing which receptor is engaged (141). Wild type LOS from strain F62 with terminal *N*-acetylgalactosamine (GalNAc) binds to "macrophage galactose-type lectin" (MGL, also present on DCs). An *lgtD* mutant with terminal galactose is recognized through an unknown receptor. While an *lgtB* mutant with terminal *N*-acetylglucosamine (GlcNAc) binds to dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN). The *lgtD* and *lgtB* deletions mirror the phenotypes that occur naturally due to slipped-strand mispairing. All of these variants have the ability to stimulate DCs to activate T cells, although LOS with a terminal GalNAc induces less IL-10 than other variants. This finding suggests that interaction with DCs may exert selective pressure for GC to vary LOS during host infection. Opa proteins, through their engagement of CEACAM1, have been shown to reduce the ability of DCs to stimulate memory responses from T cells against human immunodeficiency virus-1 (HIV-1), in part through down-regulation of the costimulatory molecule CD83 (140). It should be noted, however, that other groups have previously observed up-regulation of CD83 (and major histocompatibility complex class I, MHC-I) on DCs exposed to GC, as part of investigating how GC promotes HIV-1 infection of DCs (142). The interaction of GC with DCs is particularly interesting since DCs are critical target cells for HIV-1 infection (both pathogens share DC-SIGN as a receptor). Infections with GC have been shown to create an increased risk for HIV-1 infection through multifactorial mechanisms that remain unclear (143).

Macrophages, the next most abundant immune cell type in the fallopian tube, are primary phagocytes that are likely to encounter GC that has invaded through or between epithelial cells and entered the submucosa. Fallopian tube explants release cytokines and chemokines capable of recruiting macrophages during gonococcal infection, such as MCP-1 (CCL2) and MIP-1β (CCL4) (45). Macrophages exposed to GC produce proinflammatory cytokines such as TNFα, IL-1β, IL-6, MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), GROα (CXCL1), and CXCL10, which also likely promote PMN and T cell recruitment (144–146). Macrophages are able to phagocytose GC, with *in vitro* infections of human peripheral blood mononuclear cells (PBMCs) resulting in near complete internalization within 1 h of exposure and the killing of >60% of internalized bacteria in that same period (147). Similar results were reported for THP-1 monocytes, with >60% killing by 2 h post-phagocytosis and >80% by 5 h (146). Despite rapid eradication of the majority of the population inside PBMCs, by 6 h post-infection GC are seen persisting in macrophages. Survival occurs, in part, through the activity of the lipoprotein Ng-MIP (in the macrophage infectivity potentiator family of peptidyl-prolyl cis/trans isomerases) and by gonococcal manipulation of host gene expression to acquire necessary iron while inside (or in close association with) macrophages (146, 147). Gonococci that have been phagocytosed by macrophages are then capable of manipulating or inducing various cell death pathways. In human monocyte-derived macrophages (MDMs), intracellular gonococci are associated with increased macrophage death, with infection inducing caspase 1 and 4, indicative of pro-inflammatory (pyroptotic) cell death (148). Both THP-1 monocytes and PMBCs activate the NLRP3 inflammasome, promoting rapid cell death via pyronecrosis (145). In cultured macrophage lines, GC strain FA1090 protects U937 macrophages from staurosporine-induced apoptosis (anti-apoptotic activity), though infection induces apoptosis in the THP-1 cell line (149). Interestingly, U937 and THP-1 cell lines expressed different cytokine profiles during GC infection. For example, THP-1 cells secreted more IL-1α and IL-6, while U937 secreted more IL-10, an indication that these cell lines may have different pre-existing set points on the continuum of macrophage polarization states (150). Polarization of macrophages may itself be a relevant occurrence during infections, as primary human MDMs have been shown to polarize toward an M2 (specifically M2b) phenotype during infection with piliated, Opa^+ gonococci. This is despite the fact that cytokines expressed by these macrophages include classically M1 and M2 molecules (matching the cytokines mentioned above) (151). A similar promotion of a tolerogenic phenotype (up-regulation of IL-10 and transformaing growth factor- β, TGF-β) is observed in infections of mouse macrophages (152). Increased expression of the immunosuppressive molecule PD-L1 on human MDMs along with a reduced ability of GC-infected MDMs to stimulate $CD4^+$ T cells (151), mirrors the observations from DCs (139, 140). GC is therefore capable of manipulating both of these antigen-presenting cell types to discourage a productive adaptive immune response.

The major point of agreement between the two studies that estimated fallopian tube leukocytes is the abundance of T cells in this tissue (135, 136). In particular, the later study specifies cytotoxic ($CD8^+$) T cells as the overwhelming majority of the $CD3^+$ population in the intraepithelial compartment (1/15 ratio of $CD8^+$ cells to epithelial cells). By contrast, gamma delta ($TCR\gamma\delta^+$) T cells were observed at 1/120 and T helper ($CD4^+$) cells were observed at only 1/400 in the intraepithelial space. Natural killer T (NKT) cells with invariant Vα24-JαQ TCRα were absent. Little to nothing is known about the role of T cells in the human response to gonococci in the fallopian tube, rather, most of what is known about T cells during gonococcal infection has come from studies of mouse models of intravaginal infection. During mouse infection, GC induces cytokines that indicate the presence of a T helper 17 (Th17) cell response

(including the cytokine IL-17A) and not the cytokines expected from a Th1 response (153). In the same work, isolated mouse PBMCs and the human THP-1 cell line were both shown to make Th17-associated cytokines in response to GC infection or OMV treatment. Interestingly, blocking IL-17A or knocking out the receptor in mice, decreased neutrophil recruitment, increased recovered CFUs, and prolonged infection. These results indicate a disruption in an otherwise productive response that could clear gonococcal infection. One mechanism by which gonococci induce a Th17 response is through TLR4 recognition of gonococcal LOS, as Th17 responses are diminished in TLR4-mutant mice (154). Opa proteins then act to influence the Th17/Th1 balance, suppressing Th1 and Th2 responses (155). Central to determining the nature of the host T cell response, is the induction of TGF-β, IL-10, and type 1 regulatory T cells during gonococcal infection, which together promote Th17 responses and suppresses Th1 and Th2 responses (155, 156). Regulatory T cells (T_{regs}), including both TGF-β1-positive $CD4^+$ cells and $CD4^+$ $CD25^+$ $FoxP3^+$ cells, had been previously observed following mouse intravaginal infections (8). T_{regs} increase in number in the mouse genital tract draining lymph nodes following intravaginal gonococcal infections and TGF-$\beta1^+$ $CD4^+$ cells are observed in the mouse uterus. Though mice represent an imperfect proxy for human ascending infection and PID, the mouse model has provided valuable insight into the possible mechanistic basis of key observations in humans. Namely, why people are unable to mount a protective memory T cell response to a noticeably inflammatory bacterial infection. Future work is needed to determine the location of specific T cell subsets and their activation state during infections modeled in human tissue or *in situ* during or following natural infections.

In their long evolution with humans, gonococci have evolved methods to evade our most potent and useful defenses. GC can survive on and in epithelial cells, while facilitating passage through epithelial layers. GC is able to suppress the activity of dendritic cells and macrophages, and induce regulatory T cells to prevent $CD4^+$ T cell responses. All of this suppression occurs while triggering immune responses through TLR2, TLR4, NOD1, NOD2, TIFA, and cGAS/STING to produce an inflammatory state. When neutrophils and macrophages arrive to clear gonococci, bacteria manage to survive within these professional phagocytes and, in some cases, promote cell death. Within this cytokine/chemokine milieu, pro-inflammatory Th17 cells are induced and Th17-dependent responses act to suppress Th1 and Th2 cells that would help generate immunologic memory. To date, we have assembled a working understanding of the complement of resident immune cells and distribution of receptors in the human fallopian tube. In addition, human fallopian tube explant experiments have revealed important properties of the innate immune response to GC, while animal models have contributed information on the adaptive immune response. By combining what is known from each of these different experimental sources, we are beginning to assemble a more complete picture of how GC promotes and exploits the inflammatory environment that causes PID (**Figure 2**).

THE "STERILE" FALLOPIAN TUBE

The human female lower reproductive tract is an environment rich with commensal microbiota, containing around 10^8 bacteria per gram of vaginal fluid (157), and dominated by Lactobacillus species in the majority of women (158). Once past the cervix, however, the uterus, fallopian tubes, and ovaries were thought to be effectively sterile to protect the reproductive process. Three recent studies have utilized quantitative polymerase chain reaction (qPCR) and/or Next-Generation Sequencing (NGS) of 16S ribosomal RNA (rRNA) genes to detect bacteria in fallopian tubes (159–161). Two of these studies confirmed their findings with culture of fallopian tube-resident bacteria (159, 161). Bacteria above the cervix are indeed found at much lower abundance than below the cervix, by about four orders of magnitude. The Fallopian tube appears to contain a diverse community including members of the Gram-negative proteobacteria (Acinetobacter, Pseudomonas, Comamonas), Gram-negative anaerobes (Bacteroides), and Gram-positives (Clostridium, Enterococci, Staphylococci, Vagococcus, Proprionibacterium). Rather than being dominated by any one genus, both studies point to a more diverse polymicrobial community trending toward aerobes and facultative anaerobes that prefer slightly alkaline conditions. As with the vaginal microbiome, the microbiome analyses of the Fallopian tube do not overlap perfectly in their findings, variations that may be accounted for by geographic or ethnographic differences (one study featured 110 reproductive-age women from Shenzhen, China, another just 16 women from Brisbane, Australia).

In the lower FRT, Lactobacilli are known to prevent gonococcal establishment and colonization through multiple mechanisms. Lactobacilli keep the vaginal pH low, produce inhibitory H_2O_2 (162), compete for binding sites on epithelial cells (163), and produce surface molecules that control GC in a contact-dependent manner (164, 165). Once infection is established at the cervix and has ascended as far as the fallopian tube, it is unclear if resident microbes have any effect on the progression of inflammatory disease. The identification of a resident microbial community, however, suggests an additional variable to consider, on top of host genetics, when considering an individual's risk of negative outcomes from gonorrhea.

THINKING OUTSIDE THE TUBE

For human female upper reproductive tract infections, *ex vivo* or *in vitro* methods are the only ethical means for studying direct interactions between gonococci and their native host. Primary human-derived tissue is the nearest substitute for natural infection, but other alternatives either currently exist or could be applied to the fallopian tube given the state of the art for other tissues. Alternative technologies include human-derived primary cells, organoids (three-dimensional single or mixed cells), or organs-on-a-chip (OOC). Primary cells have been useful, to date, in the study of gonococcal infection in both males and females. Cells have been successfully derived from many of the principal tissues encountered during infection including cervical epithelium (166), endometrium (167), fallopian tube

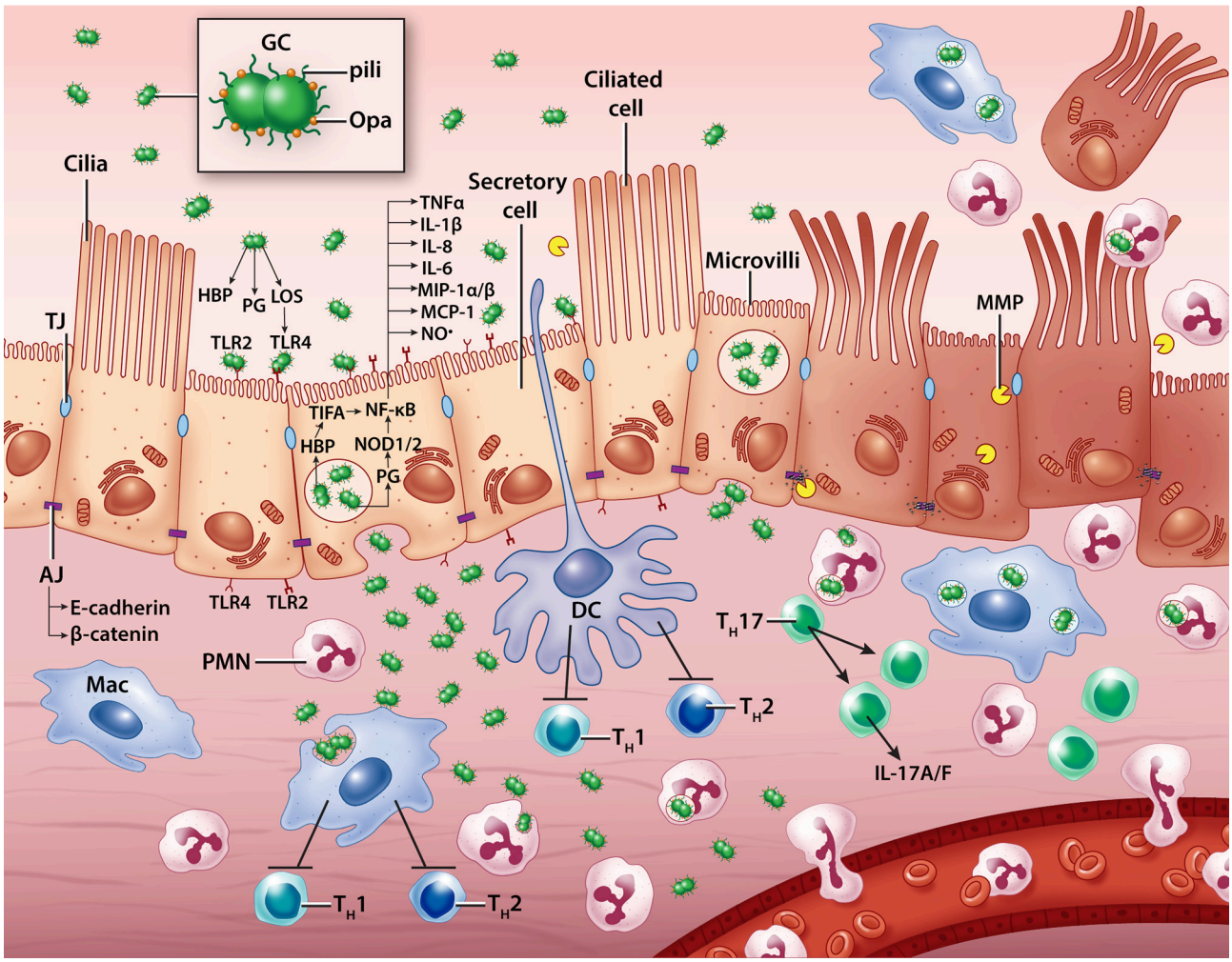

FIGURE 2 | A model of *N. gonorrhoeae* pathogenesis in the human Fallopian tube. Extracellular gonococci interact with secretory (non-ciliated) epithelial cells, inducing cytokine, chemokine, nitric oxide, and matrix metalloproteinase production. Some bacteria transcytose through epithelial cells to invade the subepithelial space where they likely encounter resident macrophages, dendritic cells, and neutrophils which can help clear infection. Immune cells are also influenced by contact with GC and the building epithelial immune response to promote pro-inflammatory T cell activity and discourage productive memory T cell responses. Ciliated cells die and are sloughed from the epithelium, possibly with the help of GC-induced host processes that disassemble cell-cell junctions. GC, gonococcus; PMN, polymorphonuclear leukocyte (neutrophil); Mac, macrophage; DC, dendritic cell; T_H, T helper cell; LOS, lipooligosaccharide; PG, peptidoglycan; HBP, heptose-1,7-bisphosphate; TLR, Toll-like receptor; MMP, matrix metalloproteinase; TJ, tight junction; AJ, adherens junction; NO•, nitric oxide.

(60), and male urethral epithelium (168). Key findings regarding differences in receptor engagement and inflammatory responses between males and females (as mentioned above) have been determined using primary cells. The principle drawback to primary cells is the loss of multicellularity characteristic of intact tissues. In tissues, multiple stratified layers may be involved in immunological cross-talk or in engaging directly with bacteria during the progression of infection. In addition, adaptation to passage in cell culture and the loss of normal development/differentiation signals can impact the performance of primary cells in culture. For fallopian tubes in particular, the mixture of ciliated and non-ciliated cells, and the differences in their behavior during infection is key to the observed pathology.

Modern methods for isolating epithelial cells from Fallopian tube have not emerged from the field of infectious disease, but rather from studies on the origins of ovarian cancer, in particular high-grade serous ovarian carcinoma (HGSOC) (169). The desire to have models for studying oncogenic transformation of fallopian tube secretory cells has led to the development of isolation methods for primary fallopian tube secretory epithelial cells (FTSECs) and immortalized lines derived from these primary cells (170). Unfortunately, a side effect of fallopian tube epithelial cell isolation is a gradual loss of ciliated cells from culture upon passage (169). Indeed, ciliated cells appear to be a terminally differentiated cell type. In the murine airway epithelium, stem/progenitor cells known as basal cells give rise to secretory epithelial cells, which can then undergo terminal

differentiation into ciliated cells (171). The maintenance of a secretory phenotype in this system is dependent on continuous signaling through the intercellular signaling receptor Notch2, without which secretory cells become ciliated cells. A similar linage relationship was also established for the epithelial cells of murine oviduct, where secretory cells are capable of self-renewal, as well as capable of differentiating into ciliated cells (172). In the oviduct, the balance of Wnt/β-catenin signaling is critical in both the maintenance of the secretory cell population and the differentiation into ciliated cells. While the human fallopian tube may not directly replicate the murine system, understanding the signals that are needed to maintain healthy mixed populations in culture allows us to improve how we establish and maintain *in vitro* models. Host developmental signaling pathways themselves may also be targets for pathogens, as evidenced by the ability of *C. trachomatis* to disrupt Wnt signaling in infected (and neighboring) epithelial cells in fallopian tube explants (100).

Defining an epithelial stem cell niche in the oviduct and determining the signals necessary to promote ciliated and secretory cell differentiation raises the possibility of using a patient's own stem cells to correct damage. Such repair could potentially restore fertility following tissue injury incurred from an STI or chemotherapy. Stem cells also provide additional possibilities for the generation of human-derived research materials. Induced pluripotent stem cell (iPSC) lines have been used to generate fallopian tube epithelium *de novo* (173). iPSCs first need to be induced to differentiate into intermediate mesoderm-like cells, and can subsequently be treated with Wnt pathway agonists (Wnt4 and Follistatin) to generate a fallopian tube epithelium that contains both ciliated and secretory cells. It is also possible to isolate multipotent mesenchymal stem cells (MSCs) from whole human fallopian tube tissue, as well as a subset of less proliferative MSCs from the mucosal portion of the tissue alone (174). These cells are capable of differentiation on adipogenic, osteogenic, and chondrogenic pathways and produce cytokines such as GM-CSF, IL-4, IL-6, TNFα and IFNγ. Kessler et al. demonstrated the isolation of adult stem cells from fallopian tube tissue, as well as defined the necessary culturing process to generate epithelia with a mixture of ciliated and secretory cells (175).

Successful culture of primary cells, stem cells, and/or iPSCs often requires suspension in (or grown on) an extracellular matrix (frequently Martigel, derived from Engelbreth-Holm-Swarm cells or minimally a collagen- or fibronectin-coated substrate). When placed in three-dimensional culture, rather than when adhered to a cell dish in monolayer, a single cell type (given proper inputs) can develop distinct polarity and cellular differentiation. These differentiated, three-dimensional cell clusters are known as spheroids or organoids. To generate faithful reproductions of fallopian tube epithelia, both iPSCs (173) and adult stem cells from dissected fallopian tube (175) need to be grown as organoids. Organoids hold tremendous promise for the study of complex cellular systems: they have the richness of signals provided by multiple cell types, spatial growth cues that monolayer cells lack, and they can be expanded in culture over long periods of time. Kessler *et al*. propagated continuously dividing, morphologically stable fallopian tube organoids for 10 months. In particular, for pathogens where animal models are lacking or do not faithfully recapitulate key aspects of infection, organoids can provide a middle ground between cell culture models and the natural infection seen in humans (176). Organoid systems have already gained traction for the study for intestinal pathogenic bacteria (177), where intestinal organoids (also known as enteroids) have been used to study bacterial adherence and invasion. For gonococci, where humans are the only natural host, the development of organoids that recapitulate the tissue architecture of fallopian tube present an exciting development and a promising future direction for host-pathogen interaction research. Since organoids can be derived from single (stem) cells, genome editing technologies including CRISPR/Cas9 could be used to alter host genes targeted during infection, prior to the growth of cells into mature organoids. These techniques have already been applied to edit the genomes of both adult stem cell- and pluripotent stem cell-derived organoids to study a variety of human diseases reviewed in (178, 179).

Another technology that could be used to replicate *in vitro* the biological complexity seen *in vivo* is organs-on-chips (OOCs), which come from a fusion of biological and engineering fields. OOCs are composed of either cell lines or primary cells from one or more tissues, cultured in custom-engineered microfluidic devices that can mimic multi-organ systems and incorporate complex biomechanical features like gas-exchange or liquid mixing (180). While OOC technology has room to improve in handling patient-derived or induced-pluripotent stem cells, microfluidic devices generally produce more reproducible results than organoids, which rely on meticulous culturing and development. Recently, a microfluidic model was constructed for the human female reproductive tract that was capable of mimicking the menstrual cycle (181). During ascending female reproductive tract infection that results in PID, gonococci must presumably interact with multiple cell types in sequence (endo/ectocervix, uterus, and fallopian tube) en route from their inoculation site. Having an interconnected, multicellular system would allow for the study of bacteria-host interactions throughout the process of gonococcal ascension to the fallopian tube. It is also unclear what bacterial or host factors predispose women to an asymptomatic cervical infection and whether this relates to their likelihood of progression to upper FRT infection. One possible contributing factor is hormonal cycles, which dramatically influence the tissue environment encountered by bacteria, and could be replicated in OOC systems. Hormonal changes are known to impact (1) primary human ectocervical epithelial cell (HECEC) responsiveness to peptidoglycan and LPS (182), (2) gonococcal survival during primary human cervical epithelial (pex) cell infection (51), and (3) the adherence and internalization rates for gonococci on fallopian tube epithelial cells (78). However, the full impact of hormonal signaling is difficult to assess in models that feature only one cell type or even one multicellular tissue. As technology evolves, more opportunities will exist to develop robust, flexible, *in vitro* models that retain the relevance of experimenting on primary human tissue.

CONCLUDING REMARKS

Neisseria gonorrhoeae remains a challenging organism for undertaking studies of host-pathogen interactions owing to its evolutionary adaptation to a life cycle in human hosts. However, the increasing utility of mouse models that incorporate human components, the availability of surgical samples from human tissues, and more adaptable human-like model systems, together provide great promise for understanding the biology of an important human pathogen. The use of fallopian tube explants provides a unique window into natural infection. Studying its multicellular epithelium has revealed how infection is linked to decreased fertility via bacteria- and host-factor toxicity to ciliated cells. Its three-dimensional structure has allowed observations about the speed of cellular invasion, the process of transcytosis and colonization of the sub-epithelial space. Thanks to insights from the fields of developmental biology, cancer biology, and gynecology, we know more than ever before about the process of ciliated cell maturation, how ciliated cells are maintained in tissues, and what immune responses are most likely to take place in the fallopian tube.

There are still several gaps that remain in our understanding of the molecular mechanisms that underlie gonococcal salpingitis. Chief among them are the cellular events surrounding ciliated cell death and extrusion from the epithelium. Prior to cell death, cellular changes result in a decrease in ciliary beat frequency (CBF). Loss of ciliated cell activity then precedes visible death and sloughing of ciliated cells. These phenomena have been difficult to measure, as the most widely accepted methods for quantitatively studying CBF involve high-speed video microscopy. Standard video microscopy is not ideal for whole tissue samples that lack optical transparency, or for monitoring changes in CBF across multiple time points in multiple samples. Many of the early works that describe decreases in CBF during gonococcal infection, and form the core of our knowledge on that topic, rely on essentially subjective visual estimates such as "percent peripheral ciliary activity (PPCA)" or "ciliary vigor" (15, 25). Future advances in live-cell, non-destructive, three-dimensional imaging of surfaces, such as light-sheet microscopy (183, 184), or methods that allow real-time *in vivo* or *ex vivo* measurements such as micro-scale tomography (185), will hopefully provide more robust measurement methods. With quantitative measurement methods, we can better probe what factors precipitate loss of CBF and what cellular mechanisms are involved in ciliated cell death and extrusion.

To understand how gonococcal infection results in permanently damaging salpingitis, we still need a clearer idea of how gonococci ascend into the fallopian tube. Current hypotheses include bacterial twitching motility, gonococcal survival inside phagocytes, or transport on sperm cells (186). We also need to understand the interplay of host factors that may predispose some women to more detrimental outcomes of ascending infection. Certainly, evidence has been presented that suggests differences in timing of infection during the menstrual cycle may influence the chance of negative outcomes. Additional investigation of host polymorphisms in immune receptors or adhesion ligands and their effects on gonococcal salpingitis represents an area for potential future investigation, especially with the increased accessibility of human genome sequencing. For example, some evidence exists that TLR2 polymorphisms and potentially other host differences can affect the outcome of *C. trachomatis* infection (187, 188). Understanding patient history, including more accurate diagnosis and documenting of instances of reproductive tract infection, is especially important now that we are aware of epigenetic mechanisms that influence future epithelial responses to repeated inflammatory stimuli (189, 190). At the center of any discussion of how to model infectious diseases should be a clear understanding of how the knowledge we gain into basic biological mechanisms translates into the features we see (or seek to prevent) in human disease. Fallopian tubes, and other human tissues, provide an excellent model system for answering certain questions about how gonococci interact with, and provoke immune responses from diverse human genetic backgrounds. Along with cellular and whole-animal systems, organ cultures represent a vital piece of the research puzzle that together allows us to assemble a more complete picture of *N. gonorrhoeae* pathogenesis.

AUTHOR CONTRIBUTIONS

JL and JD conceived and outlined the manuscript. JL wrote the manuscript and designed figures. JD edited the manuscript and figures.

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REVIEW
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Intimate Relations: Molecular and Immunologic Interactions Between *Neisseria gonorrhoeae* and HIV-1

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While the global incidence of human immunodeficiency virus (HIV-1) remains well above UNAIDS targets, sexual transmission HIV is surprisingly inefficient. A variety of host, viral and environmental factors can either increase HIV-1 shedding in the infected partner and/or increase mucosal susceptibility of the HIV-1 uninfected partner. Clinical and epidemiological studies have clearly established that *Neisseria gonorrhoeae* substantially enhances HIV-1 transmission, despite it not being an ulcerative infection. This review will consider findings from molecular, immunologic and clinical studies that have focused on each of these two human-restricted pathogens, in order to develop an integrative model that describes how gonococci can both increase mucosal shedding of HIV-1 from a co-infected person and facilitate virus establishment in a susceptible host.

Keywords: *Neisseria gonorrhoeae*, HIV-1, infectious synergy, co-infection, sexually transmitted infection, sexually transmitted disease

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INTRODUCTION

It is remarkable to consider that human immunodeficiency virus type 1 (HIV-1) has caused a global pandemic despite being quite poorly transmissible, with the chance of an HIV-infected, treatment-naive individual passing the virus to their partner through penile-vaginal sex being a fraction of one percent (Royce et al., 1997; Galvin and Cohen, 2004). The explanation for this apparent contradiction is that multiple factors can enhance the likelihood of transmission, including the type of mucosa exposed to virus during sex, the viral titer in the anogenital secretions of the infected partner, and the presence/number of CD4 receptor-expressing target cells in the mucosa of the uninfected partner (Royce et al., 1997; Ferreira et al., 2014). When viewed through this lens, it becomes clear why sexually transmitted infections (STIs) might facilitate HIV-1 transmission, either directly through their effect on viral replication or indirectly through the inflammation-mediated recruitment of lymphocytes and/or disruption of the mucosal barrier. While a variety of co-infections can influence HIV-1 transmission (Fleming and Wasserheit, 1999; Kaul et al., 2008), this review will focus on the effects of *Neisseria gonorrhoeae*, both because gonococcal infection markedly enhances HIV-1 transmission at an epidemiological level (Fleming and Wasserheit, 1999; Cohen, 2012) and because a number of studies have explored the molecular and immunologic aspects of this synergy. When these studies are considered together, the complexity of the association between these pathogens starts to become clear, even though there are apparently contradictory findings depending upon the models used to explore this pathogen interaction. Herein, we will review the replication cycle of HIV-1 and consider the possible impact

of *N. gonorrhoeae* infection. Then, we will attempt to integrate these research findings to generate a model that can be used to understand the outcome of this common co-infection, providing a framework to direct future research and a guide for ongoing attempts to intervene.

HIV-1 INFECTION

Despite significant global efforts to control HIV-1 transmission, the virus continues to be a scourge, particularly in high risk populations that include men who have sex with other men (MSM) and female sex workers. Acute HIV-1 infection is characterized by a transient period of very high titer viremia that lasts for just a few weeks, which is followed by a chronic infection phase characterized by a much lower viral load in both blood and anorectal secretions once a host adaptive immune response has been generated. However, HIV-1 infection is lifelong, due in equal parts to the virus's ability to integrate its genome into the chromosomal DNA of long-lived CD4+ memory T cells where it can reside undetected, while simultaneously evading the adaptive response during active replication by mutating its genome at a phenomenal rate [4×10^{-3} per base per cell, the highest for any described biological entity (Cuevas et al., 2015)]. For the latently infected cell, subsequent immune activation driven by infectious or other inflammatory stimuli will cause the virus to exit latency and initiate active replication resulting in viral propagation and eventual death of the infected cell. Over an average of 8–10 years, a combination of active cellular viral replication and bystander CD4+ T cell death leads to gradual depletion of blood CD4+ T-cell numbers, along with persistent immune activation and CD8+ T cell exhaustion and dysfunction, with irreparable damage to the structure of secondary lymphoid organs preventing recovery of depleted CD4+ T-cell counts. Once the blood CD4+ T cell count falls below ∼200 cells/ml, this is now classified as acquired immunodeficiency syndrome (AIDS), and the immune system is now unable to protect the infected individual against a variety of opportunistic infections and neoplasms [for review see Langford et al. (2007)].

Fortunately, HIV-1 is now a manageable chronic infection thanks to the development of antiretroviral compounds that target multiple different points of the viral life cycle. Combination antiretroviral therapy (cART) utilizes a combination of several of these compounds (generally 2–3 separate medications) to simultaneously block multiple stages of the viral life cycle and permit recovery of the immune system. Successful treatment suppresses virus replication to virtually undetectable level, restoring an individual's life expectancy to near-normal (Arts and Hazuda, 2012). However, while effective at preventing HIV-1 replication, these medications are not a cure since the latent HIV reservoir remains unaffected by the treatment. Because of this, interruption of therapy consistently and predictably leads to the rebound of viral replication in blood, and the patient will progress to AIDS if medication is not continued. However, while the infected individual cannot be cured, the remarkable efficacy of cART is such that HIV-infected individuals who are compliant with their medication and maintain a suppressed viral load are considered to be non-infectious to their sexual partners (Cohen et al., 2016; Rodger et al., 2019).

By halting transmission, cART has contributed to a slow reduction in the global incidence of new HIV-1 infections (UNAIDS, 2019). However, the administration of therapy is costly, and some HIV-1 infected individuals do not have access to these drugs – this is particularly true within marginalized key populations such as MSM and female sex workers (Levi et al., 2016; Bain et al., 2017). According to UNAIDS (UNAIDS, 2019), there were 37.9 million people living with HIV-1 globally in 2018, with 23.3 million (61%) of these individuals on antiretroviral therapy. Indeed, the combination of ongoing HIV transmission by untreated (or inappropriately treated) individuals, together with the greatly enhanced survival of infected individuals taking antiretroviral treatment (ART), means that there is a steady increase in the global prevalence of HIV-1 each year; globally, with a 23% increase between 2010 and 2018 (UNAIDS, 2019). The clear message is that the only way to reduce the global burden of HIV-1 is to develop new and better interventions to reduce HIV-1 transmission and/or to develop feasible strategies for HIV cure.

HIV-1 TRANSMISSION

Human immunodeficiency virus type 1 transmission risk varies greatly between populations, with heterogeneity linked to multiple behavioral and biological factors that include socioeconomic status, race, sexual partner numbers and patterning, mucosal site of exposure, circumcision status and contraceptive use, the presence of anogenital infections and the mucosal microbiome. Exposure of the anorectal mucosa to HIV through receptive anal intercourse remains the highest risk sexual behavior for HIV-1 transmission (Patel et al., 2014), with an incidence that averages 138 per 10,000 exposures. In comparison, the rates of transmission during penile-vaginal sex are approximately ten-fold lower, averaging 8 and 4 cases per 10,000 coital acts for male-to-female and female-to-male transmission, respectively (Patel et al., 2014). However, clinical and epidemiological studies have clearly established that HIV-1 transmission risk is substantially and consistently increased in the presence of certain co-infecting bacterial, viral and fungal pathogens (Galvin and Cohen, 2004) or inflammatory mucosal microbiota (Atashili et al., 2008; Mirmonsef et al., 2012; Schellenberg et al., 2012). In each case, the mucosal immune milieu is altered in response to these microbial challenges in a way that damages epithelial integrity and enhances HIV-1 shedding (in the infected partner) and access to preferred viral target cells in the uninfected partner, namely activated mucosal $CD4^+$ helper T-cells.

In considering how anogenital co-infections may influence HIV-1 transmission, it is important to differentiate between effects that alter the *infectiousness* of the infected partner versus those that influence the uninfected partner's *susceptibility* to infection (Galvin and Cohen, 2004). Given that the HIV-1 titer in genital secretions dictates the likelihood of transmission from an infected person (Quinn et al., 2000), infectiousness will be increased by any factor that increases virus shedding in genital

mucus or semen, or perhaps that selects for viral variants that are inherently better able to establish infection. Synergistically in the uninfected partner, susceptibility to infection will be increased by any host-intrinsic factors that provide portals of entry across the mucosal barrier, since this provides access to target CD4+ T cells in the underlying tissues, or that stimulate an immune response that is either ineffective at combatting or, even worse, that promotes HIV-1 replication (Galvin and Cohen, 2004). For example, a co-infecting pathogen may increase an HIV-infected person's infectiousness by stimulating an immune response that recruits virally infected cells to the genital tract and/or by increasing viral production by HIV-infected cells that are already resident there (**Figure 1**). Alternatively, mucosal tissue barrier function may be hampered by direct pathogen-induced cytolysis of epithelial cells and/or indirectly by the disruption of epithelial cell junctions in response to inflammatory cytokines elicited by a co-infecting pathogen, increasing an individual's susceptibility by allowing HIV-1 entry into the underlying tissues (**Figure 2**).

HIV-1 BIOLOGY

While the intact mucosa typically provides an effective barrier against HIV-1, the virus may normally gain access to underlying target cells via its transcellular transport from the mucosal lumen into the lamina propria through intact epithelial cells, may pass through an non-intact epithelium that has been disrupted by inflammation or by physical stress during sexual activity, or may be taken up by tissue resident dendritic cells (DCs) in the lamina propria as they extend dendrites between epithelial cells to sample the luminal microenvironment for foreign antigens (Tugizov, 2016). Once within the mucosal tissues, activated CD4+ T cells are the primary target for HIV-1 infection. However, other cell types can either directly or indirectly facilitate infection. Most notable in this regard, DCs may express DC-SIGN, a cell surface-expressed receptor that binds the HIV-1 envelope glycoprotein, gp120 (Geijtenbeek et al., 2000). This interaction does not lead to direct DC infection, but the DC may transfer the virus to T cells with which it engages *in trans*, either within the mucosal tissue microenvironment or after the DC migrates into secondary lymphoid organs (Shen et al., 2009). Macrophages can also take up cell-free virus and present it to CD4+ T cells. While macrophages are considered to be intrinsically resistant to HIV-1 infection, they clearly can be infected by HIV-1 since integrated virus is apparent and has been demonstrated within them (Araínga et al., 2017; Clayton et al., 2017). The contribution of these cell types to infection, and the effect that *N. gonorrhoeae* may have on HIV exposure and the outcome of this interaction, will be discussed further below.

Upon contact with CD4+ T cells, the homotrimeric HIV-1 envelope protein (Env) engages cell surface-expressed CD4 and one of its co-receptors, generally either the CXCR4 or CCR5 chemokine receptor, depending on the viral variant's tropism. This interaction promotes a dramatic conformational change in Env to drive fusion of the HIV-1 and target cell membranes, allowing entry of the viral core into the cytoplasm (Rawle and Harrich, 2018). Once uncoated, the single-stranded RNA genome is reverse transcribed to double-stranded DNA by the pre-formed viral reverse transcriptase; this is a prerequisite for viral genome transport into the nucleus and its integration into the host cell genome. Integration allows establishment of a durable yet transcriptionally silent viral reservoir within long-lived memory T-cells (Finzi et al., 1997; Churchill et al., 2015). However, cellular activation promotes HIV-1 exit from this latent state, driven in part by the T cell receptor-stimulated translocation of active human nuclear factor-kappa B (NF-κB) into the nucleus, where it binds the HIV-1 long terminal repeat (LTR) to initiate transcription of the integrated proviral genome (Mbonye and Karn, 2017). In this context, it is notable that NF-κB is activated by a wide variety of stress responses and danger signals, including by cellular exposure to certain inflammatory cytokines or microbial-associated molecular patterns (MAMPs; also known as pathogen-associated molecular patterns, or PAMPs) (Oeckinghaus et al., 2011; Hayden and Ghosh, 2012). This suggests a variety of mechanisms by which co-infection might promote HIV-1 infection.

Neisseria gonorrhoeae INFECTION

All STIs have been demonstrated to enhance HIV transmission to some degree, and the epithelial disruption induced by ulcerative STIs such as syphilis provides an obvious mechanism. Among STIs that can increase HIV-1 transmission, however, *N. gonorrhoeae* seems to have a disproportionately large effect on HIV transmission, particularly when considering that it is not an ulcerative infection (Fleming and Wasserheit, 1999; Cohen, 2012). This could be mediated via enhancement of HIV infectiousness in HIV-infected (**Figure 1**) and/or HIV susceptibility in uninfected individuals (**Figure 2**). In terms of the former, HIV-1 infected men with gonorrhea were observed to shed ∼10-fold more virus in semen than do men without urethritis, an effect greater than that caused by other non-ulcerative STIs (Cohen et al., 1997). Notably, *N. gonorrhoeae* infection had no impact on viral titers in blood, since these were not different between *N. gonorrhoeae* and HIV-1 co-infected versus HIV-1 alone control groups. However, appropriate and specific antibiotic therapy reduced semen viral shedding in the gonococcal-infected individuals to levels similar to gonorrhea-uninfected controls, suggesting that this was a localized effect that directly correlates with the mucosal gonococcal infection. The global effect of this relationship on HIV-1 spread is compounded by the fact that ∼87 million new gonococcal infections occur each year (Rowley et al., 2019), allowing frequent opportunity for co-exposure in sexually active or at risk individuals.

The gonococcus is an obligately human-restricted pathogen that is highly adapted to colonize mucosal tissues, preferentially the male urethra and female endocervix, but it can also effectively colonize the nasopharynx, rectum and conjunctiva when these tissues become exposed (Britigan et al., 1985; McSheffrey and Gray-Owen, 2015; Unemo et al., 2019). The outcome of gonococcal infection varies, ranging from an absence of any clinical signs to an intensely pathogenic inflammation manifesting as a purulent discharge composed almost entirely

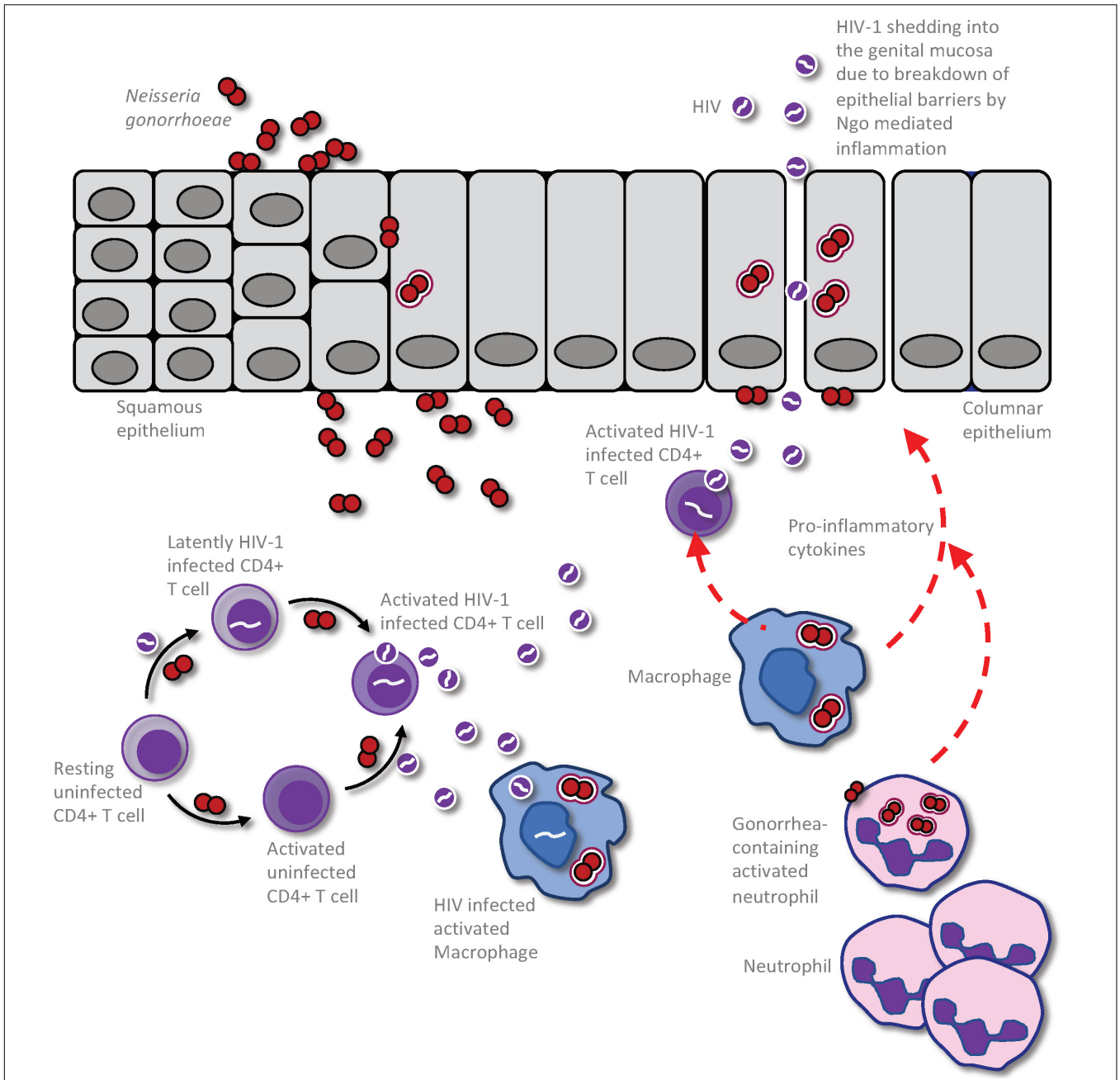

FIGURE 1 | Impact of *Neisseria gonorrhoeae* infection on HIV production and shedding in an already HIV infected person. The presence of the gonococci within the urogenital tract results in an exuberant neutrophil response that is characterized by increased presence of pro-inflammatory cytokines and chemokines. This results in the recruitment of infected CD4 T-cells to the area of gonococcal infection, leading to activation of the latent HIV and release of virions to the genital microenvironment. This event could also result in infection of HIV-uninfected but gonococcal-activated CD4 T-cells and further propagation of the virus within the patient. Macrophage and dendritic cells (DCs) may also take up the virus to be presented to CD4 T-cells in distal lymphoid organs, increasing the efficiency of CD4 T cell infection and causing further propagation of infection, ultimately leading to destruction of secondary lymphoid organs. Following gonococcus-induced disruption of the epithelial barrier, either directly by the bacterium or through the inflammatory milieu, the cell free HIV particles may be released freely into the genital lumen to increase infectious potential of this HIV infected individual.

of polymorphonuclear neutrophils (Criss and Seifert, 2012). These differences in outcome may depend, in part, on the physiology, tissue structure and innate immune effectors resident within the infected tissues (Edwards and Apicella, 2004; Unemo et al., 2019), since male urethral infections tend to be symptomatic while female endocervical infections are less frequently so; however, asymptomatic infections can occur in men (Handsfield et al., 1974; Kent et al., 2005) and cervicitis

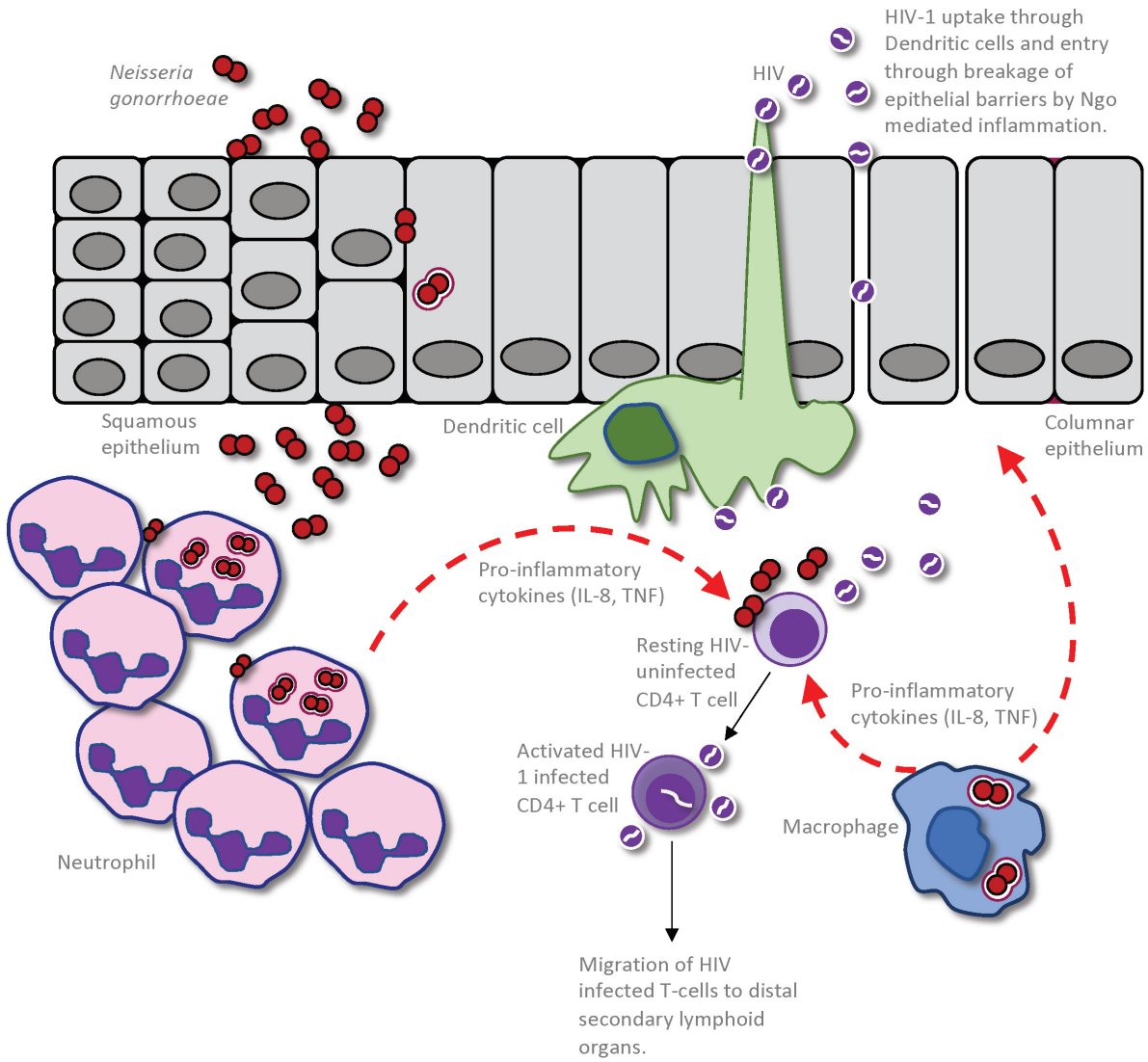

FIGURE 2 | Impact of *Neisseria gonorrhoeae* infection on establishment of HIV infection in a seronegative individual. The gonococcus may cause an increased susceptibility to HIV infection through a combination of the inflammation-induced damage of the epithelial barrier and increased inflammation within the genital microenvironment. This allows enhanced entry of HIV into the submucosa, either through dendritic cells (DCs) that are sampling the mucosal environment or through disrupted epithelium. Given that the gonococci cause an intense localized inflammation, as evidenced by an extensive neutrophil influx to the site of infection, CD4 T-cells that are recruited to the site of infection will be activated by the cytokines and other inflammatory mediators so as to become optimal targets for HIV infection and replication. Macrophage that are recruited to the site of gonococcal infection may also produce pro-inflammatory cytokines and chemokines to simultaneously activate local CD4 T-cells and recruit further HIV-receptive target cells. Furthermore, the macrophages or DCs can take up the HIV present in the genital microenvironment and travel to distal secondary lymphoid organs where they can further promote HIV infection of CD4 T-cells in these regions.

does occur in a substantial proportion of women (Unemo et al., 2019). Moreover, gonococcal-associated pathogenesis becomes particularly devastating when the infection ascends into the female endometrium and fallopian tubes, where it can trigger pelvic inflammatory disease (PID) with intense inflammation leading to tissue scarring, chronic pain and infertility (Taylor et al., 2011; Lenz and Dillard, 2018). In humans, gonococcal PID tends to onset with abrupt and intense symptoms, often during the first 10 days after the onset of menses. Sex hormone cycling, which affects both the physiology and microbiome of the female genital tract (Bradley et al., 2018), also impacts the outcome of uterine infection in a mouse model of pelvic inflammatory disease, with a marked increase in gonococcal tissue invasion and inflammation during the progesterone-dependent diestrus stage of the reproductive cycle (Islam et al., 2016). How much these differences are explained by hormonal effects on gonococcal physiology (James and Swanson, 1978; Salit, 1982) versus the physical and immunological integrity of the mucosal barrier (Islam et al., 2016; Lenz and Dillard, 2018) remains unclear.

N. gonorrhoeae PATHOGENESIS

Studies that have attempted to understand the impact and pathogenesis of *N. gonorrhoeae* in the context of HIV-1 co-infections are inherently challenging because both pathogens are human restricted. Our understanding of how the gonococci contribute to HIV-1 transmission must, therefore, be inferred by combining the insights gained through clinical surveillance of gonococcal and HIV-1 co-infections with those from laboratory-based models developed to study one or the other pathogen. The remainder of this review will bring these results together to develop an integrated model of how *N. gonorrhoeae* impacts HIV-1 shedding and susceptibility to HIV-1 infection, gaining insights from studies exploring the molecular and immunological processes that govern aspects of gonococcal infection with potential to impact upon HIV-1, as well as clinical studies exploring the impact of *N. gonorrhoeae* co-infection on an individual's susceptibility to HIV-1 or their likelihood to transmit the virus to their partners.

Cellular Attachment

Neisseria gonorrhoeae are not overtly pathogenic in that they do not produce protein-based exotoxins or cause direct host cellular killing, consistent with the fact that infections are often asymptomatic. Pathogenesis is, therefore, a result of neisserial replication within the tissues. To establish infection, the gonococci attach to the epithelia via their type IV pilus, which retracts to allow the bacteria to move through the mucus and establish tight secondary binding between outer membrane protein adhesins on the bacterial surface and their cognate host cellular receptors (Virji, 2009; Lenz and Dillard, 2018). Of these, the gonococcal colony opacity-associated (Opa) proteins have been shown to attach to members of the human carcinoembryonic antigen-related cellular adhesion molecule (CEACAM) family of surface glycoproteins, with the different binding specificities of each Opa protein variant and the varying expression of different CEACAMs on each cell type ultimately determining the outcome of this interaction (Sadarangani et al., 2011; Islam et al., 2018; Yu et al., 2019). Transgenic mouse-based studies have established that Opa protein binding to CEACAM1 and/or CEACAM5 promotes epithelial attachment in the upper and lower female genital tract, respectively (Islam et al., 2018), while Opa binding to the neutrophil-expressed innate decoy receptor CEACAM3 promotes gonococcal engulfment and killing, and elicits a self-propagating pro-inflammatory cytokine response that drives the ongoing recruitment and activation of neutrophils to the infected tissues (Sintsova et al., 2014; Islam et al., 2018). Aside from these direct effects, CEACAM binding also elicits an integrin-dependent increase in epithelial cell affinity for the extracellular matrix, which reduces exfoliation of the infected cell so as to promote infection (Muenzner et al., 2010). The cumulative benefit of these effects presumably explains why bacteria recovered from clinical specimens tend to be expressing one or more of the phase variably-expressed Opa protein variants (James and Swanson, 1978; Swanson et al., 1988; Jerse et al., 1994).

While CEACAM-dependent binding has been the most extensively characterized means of tissue attachment, Opa-independent interactions, including endocervically expressed CR3 binding by the gonococcal pilin-linked glycans (Jennings et al., 2011) and urethrally expressed asialoglycoprotein receptor binding to gonococcal surface glycans (Harvey et al., 2001), can also promote tissue association and affect a cellular response. A key challenge in understanding how the mucosal tissues respond to *N. gonorrhoeae* and, thereby, how co-infection influences HIV transmission, stems from the fact that these and other gonococcal surface ligands are both phase and antigenically variable, allowing stochastic on-off switching of their expression and alteration of the bacteria's epitope repertoire and phenotype, respectively (Virji, 2009). Thus, a gonococcal population may elicit different effects depending upon which adhesins (and other surface antigens) are expressed, and what cellular receptors are expressed by the infected tissues. One must remain mindful of this when interpreting laboratory-based experiments, but the contribution of this variability to the different outcomes of natural infection must also be considered since each niche will presumably select for the most fit phenotype in an ongoing basis.

Tissue Response

The specificity of neisserial adhesins for human-derived receptors has led to considerable effort toward modeling molecular aspects of infection using immortalized cell lines, primary cells, and tissues. Classical fallopian tube organ culture studies revealed that gonococci penetrate through non-ciliated epithelia via a transcellular route to emerge into the sub-epithelial spaces (McGee et al., 1983), and caused the sloughing of adjacent (but uninfected) ciliated cells (Melly et al., 1984). More recently, primary human cervical explants have been used to reveal that infection with gonococci that do not express Opa proteins promotes dissociation of intercellular junctions to allow luminal shedding of the infected cells, while epithelia remains intact when the bacteria express CEACAM-specific Opa variants (Wang et al., 2017; Yu et al., 2019), consistent with the effects seen in human CEACAM-expressing transgenic mouse-based studies (Muenzner et al., 2010). When using ureter-derived tissue as a model of male urethral infection, luminally exposed cells that engulf piliated (but non-CEACAM-binding) gonococci were also observed to release their intercellular junctions and be shed, thinning the stratified epithelia and exposing underlying tissues (Mosleh et al., 1997). Considered together, these data suggest that Opa-dependent binding to epithelial-expressed CEACAMs allows attachment and impedes the exfoliation that normally occurs as a defense against bacterial infection, thereby promoting gonococcal infection by maintaining the epithelial integrity. It is notable in this regard that the transitional zone between the ectocervix and endocervix displays very little CEACAM expression and allows gonococcal penetration regardless of their Opa protein expression (Yu et al., 2019), suggesting that this region may be particularly susceptible to gonococcal-induced epithelial thinning to promote exposure to underlying HIV-1 target cells.

Aside from the direct effects of *N. gonorrhoeae* on the epithelium, the inflammatory response to infection can itself

interrupt the epithelial barrier. The gonococci have a curious penchant for stimulating inflammation by virtue of their ongoing release of peptidoglycan-derived NOD1 agonists (Lenz et al., 2017), heptose phosphate metabolites that activate the AlpK1-TIFA innate sensor pathway (Gaudet et al., 2015), outer membrane "blebs" containing abundant TLR4 [endotoxin (Pridmore et al., 2003)] and TLR2 [lipoproteins (Fisette et al., 2003), PorB (Zhu et al., 2018)] agonists, and the type IV secretion system-mediated release of TLR9-stimulatory single-stranded chromosomal DNA (Ramsey et al., 2011) into the extracellular milieu. The release of these microbial-associated molecular pattern (MAMP)-containing metabolites is energetically wasteful, implying that the resulting inflammation somehow facilitates gonococcal infection, such as by allowing increased nutrient leakage into the mucosal tissues or by skewing the innate and/or adaptive immune responses so that infection can persist. Regardless, in the context of co-infection with HIV-1, pro-inflammatory cytokines such as TNF are abundantly produced upon gonococcal infection of human fallopian tube cultures, and promote cellular apoptosis and a breach in epithelial integrity (Morales et al., 2006), as well as increased HIV-1 expression in infected CD4+ T cells (Ferreira et al., 2011).

Beyond the effects of *N. gonorrhoeae* on HIV transmission, it is also interesting to consider that HIV-1 may influence the outcome of gonococcal infection. Inflammatory cytokines produced during the acute phase of HIV-1 infection cause a direct loss in epithelial integrity within the intestinal mucosa (Nazli et al., 2010), allowing bacteria and bacterial products to pass into the tissues and cause systemic inflammation that drives further viral replication (Brenchley et al., 2006). Other studies have shown that interaction with HIV-1 envelope protein gp120 may itself disrupt tight and adherens junctions, causing permissiveness to infection by other viral and bacterial pathogens (Nazli et al., 2010; Tugizov, 2016). This would suggest that a synergistic effect may occur, where the inflammatory response to HIV-1 would allow increased gonococcal tissue penetration, which could recruit cellular targets of HIV-1, further disrupt the epithelial barrier, and drive NF-κB-dependent HIV replication. If inflammation and/or tissue penetration are, indeed, of benefit to the gonococci, then this may facilitate infection. However, to our knowledge, there are not yet clinical or epidemiological studies to determine whether chronic HIV-1 infection promotes gonococcal infection or disease.

GONOCOCCAL-SPECIFIC EFFECTS ON HIV-1 TARGET CELL POPULATIONS

The hallmark of a gonococcal infection is the pathogenic recruitment of neutrophils to the site of infection, leading to a purulent discharge that results from this prolonged and inappropriately exuberant neutrophil recruitment. Despite this intense inflammatory response, there is curiously little evidence of an adaptive response to gonococcal infection. The answer to this conundrum appears to stem from the Th17-biased response to gonococcal infection, at least in the case of lower genital tract infection in female mice. Specifically, gonococcal infection leads to marked expression of the Th17-related IL-17 and IL-22, without any concomitant appearance of Th1 or Th2 cytokines, or of gonococcal-specific antibodies. When IL-17 signaling was blocked with IL-17-specific antibodies or in IL-17 receptor-deficient mice, neutrophil recruitment decreased and the gonococcal burden increased, implying that IL-17-driven effects combat the infection (Feinen et al., 2010). However, strikingly, the administration of TGFβ-specific antibodies during primary gonococcal infection effectively inhibited the Th17 response (because TGFβ drives Th17 differentiation in mice) but allowed the emergence of a gonococcal-specific adaptive response that protected against re-infection (Liu et al., 2012). A protective memory response could also be elicited by blocking IL-10 signaling (Liu et al., 2014) or by the administration of microencapsulated IL-12 (Liu et al., 2018) during the primary infection. Considered together, these studies suggest that the gonococci have evolved to persist during the Th17-driven inflammation, with the benefit being that this highly polarized response prevents the individual from becoming immune to the infection. What remains unclear is whether this Th17 response is an inherent bias of the female genital tract or whether the gonococci have properties that elicit this outcome, such as through their ongoing and profuse release of MAMPs discussed earlier in this review. Regardless, this effect may directly influence HIV-1 transmission because activated Th17 cells are the primary target for this virus during acute mucosal infection (Gosselin et al., 2010; Prendergast et al., 2010; Alvarez et al., 2013; McKinnon et al., 2015), and may indirectly affect virus susceptibility if HIV-specific adaptive responses were to be inhibited.

Innate Activation of HIV-Infected CD4 + T Cells

The disparate effects that CD4+ T cells may have on host immune protection, exemplified by the opposing outcomes of Th17 and Th1 effector responses on gonococcal infection discussed above, makes it somewhat foolhardy to try and difficult to extrapolate from cell-based *in vitro* studies to predict the global outcome of infection *in vivo*, particularly considering the central role that these cells play as both a cellular HIV target and drivers of host immunity against HIV infection. Nevertheless, it is reasonable to consider the effect of co-infection on a cellular level. Given that NF-κB-driven transcription is the most dramatic outcome of cellular activation in response to microbial-derived immune agonists, and that this transcription factor governs expression from the HIV-1 LTR, this is an obvious potential link between co-infection and HIV-1 replication. As noted above, pro-inflammatory cytokines expressed by gonococcal-infected genital epithelial cells provoke LTR-driven HIV expression in an NF-κB-dependent manner (Ferreira et al., 2011), revealing an indirect effect that should be shared by any inflammatory stimuli. A more direct effect on HIV-1 replication is apparent with gonococcal-derived TLR2 agonists, which promote HIV-1 uptake and nuclear import of viral DNA in resting primary human CD4+ T cells (Ding et al., 2010). Notable in this regard, gonococcal-derived endotoxin (a TLR4 agonist) does not have

a similar effect, consistent with the fact that most other TLRs are not expressed by human CD4+ T cells (Ding et al., 2010; Malott et al., 2013).

Once latent HIV-1 infection has been established, gonococcal infection will elicit a robust viral LTR-dependent transcriptional response. This effect occurs independent of any TLR (or NOD) receptors, and can be elicited by simple exposure of the latently infected CD4+ T cells to bacterial-free culture supernatants from *N. gonorrhoeae*, but does not occur in response to culture supernatants from non-neisserial bacterial species (Chen et al., 2003; Malott et al., 2013). This activity is due to the peculiar propensity for pathogenic and commensal *Neisseria* species to release heptose phosphate-containing metabolites from the ADP-heptose pathway, a biosynthetic cascade that normally leads to the incorporation of heptose into the inner core of gram negative lipopolysaccharides (Malott et al., 2013; Gaudet et al., 2015); other bacteria effectively sequester heptose phosphates within their cytoplasm (Gaudet et al., 2015). These 7-carbon phosphorylated sugars activate innate immune responses in mammalian cells independent of canonical pattern recognition receptor-mediated signaling. Instead, they are directly bound by the cytosolic alpha kinase 1 (AlpK1). Heptose phosphate binding activates the kinase so that it can phosphorylate TIFA, which then self-associates into large cytosolic TIFAsome structures that function as a scaffold to drive NF-κB-mediated transcription (Gaudet et al., 2015; Milivojevic et al., 2017; Adekoya et al., 2018; Zhou et al., 2018). Heptose phosphates therefore provide a direct link between gonococcal infection and HIV-1 replication in CD4+ T cells by the virtue of NF-κB activation through TIFA-AlpK1 axis.

CEACAM1-Mediated Suppression of T Cell Responses

While innate drivers of inflammation stimulate HIV-1 transcription, gonococcal Opa protein binding to human CEACAM1 can broadly suppress CD4+ T cell responses to T cell receptor engagement and certain other activating stimuli (Boulton and Gray-Owen, 2002). Very little CEACAM1 is apparent on resting lymphocytes, but its expression is upregulated upon cellular activation. Unlike other immune cells, CD4+ T-cells are unable to engulf gonococci that attach to their surface, leading to a stable association between Opa protein-expressing gonococci and CEACAM1 on the cell surface (Lee et al., 2008). When the T cell receptor becomes engaged by antigen presenting cells, it would normally unleash a kinase-dependent signaling cascade that activates the T cell. However, when CEACAM1 is engaged by gonococci (Lee et al., 2008) or gonococcal-derived outer membrane blebs (Lee et al., 2007), tyrosine residues within the immune receptor tyrosine-based inhibitory motif (ITIM) in the CEACAM1 cytoplasmic domain also become phosphorylated, creating docking sites to recruit SHP-1 and SHP-2 phosphatases (Boulton and Gray-Owen, 2002; Gray-Owen and Blumberg, 2006; Lee et al., 2008). Once activated, these enzymes rapidly dephosphorylate the T cell receptor and downstream signaling effectors, shutting down the activating response (Boulton and Gray-Owen, 2002; Lee et al., 2007). This immune inhibitory effect is compounded by the fact that Opa protein binding to CEACAM1 on DCs reprograms their maturation such that they are not able to effectively present antigen to T cells (Yu et al., 2013). In the context of HIV-1 co-infection, the hampering of T cell responses may have two different effects. First, given that the nuclear localization of NF-κB is a primary outcome of T cell receptor engagement, it seems reasonable to assume that the phosphatase activation will increase the threshold of cellular activation required to drive HIV-1 from latency. Second, the central role that CD4+ T cells play in the adaptive immune response suggests that CEACAM1 binding may suppress development of an adaptive response to the infection. While the reduced inflammation and absence of memory response may both facilitate gonococcal infection, their cumulative effect on HIV-1 infection and immunity are difficult to test given that gonococcal Opa proteins only bind human (and not mouse or other) CEACAM1.

Gonococcal Interaction With Antigen Presenting Cells

Macrophage and DCs reside as sentinels within the submucosa to detect microbes infiltrating the otherwise sterile tissues, and then direct the immune responses to eliminate the invading pathogens. After capture of microbial-derived products, they migrate to secondary lymphoid organs to present foreign epitopes to T cells and thereby elicit a pathogen-specific adaptive response. Both phagocyte types express CD4 and the HIV-1 co-receptors, CXCR4 and CCR5, which facilitate viral uptake (Martin-Moreno and Munoz-Fernandez, 2019). However, while macrophages may be productively infected in certain contexts, DCs express innate viral restriction factors so that they rarely become infected, and produce relatively little virus when they do (Smed-Sorensen et al., 2005). Despite this fact, DCs are key to the establishment of infection, in part because they can project dendrites between epithelial cells and come in contact with virus as they sample the mucosal lumen, facilitating viral transport across an otherwise intact epithelial barrier (Martin-Moreno and Munoz-Fernandez, 2019). Further exacerbating this effect, HIV-1 binding to DC-SIGN facilitates its retention on the dendritic cell surface (Geijtenbeek et al., 2000), allowing the virus to be carried to the lymph nodes and then transferred to CD4+ T cells as they engage with the phagocyte (Martin-Moreno and Munoz-Fernandez, 2019). Events that promote macrophage or dendritic cell sampling and/or movement to the lymph nodes, as might occur during gonococcal co-infection, would presumably facilitate this process.

While tissue resident DCs may resist infection, there is some indication that exposure to certain stimuli might affect this outcome. For instance, *in vitro* studies indicate that gonococcal co-infection of monocyte-derived DCs increases their likelihood for HIV-1 infection based upon the emergence of virally derived reverse transcriptase activity, and this effect can be reproduced through the administration of purified gonococcal PorB or lipoproteins, both of which are TLR2 agonist, or peptidoglycan (Zhang et al., 2005). This study did not correlate cellular phenotype with viral expression on a single cell level, and did not quantify the proportion of cells that became infected or

number of viral particles produced by the human cells, so how productive the infections were and whether the virus-expressing cells were actually DCs remains unclear. Notably, a separate study confirmed that TLR2 agonists enhanced HIV-1 production from human DCs, but also observed that TLR4 agonists instead suppressed HIV expression by virtue of their distinctive ability to stimulate production of type-I interferons, which upregulate cell autonomous immune defenses (Thibault et al., 2009). Given that this study used purified agonists, it remains unknown how the combination of these and other bacterial-derived innate agonists might combine to influence HIV-1 replication *in vivo*.

Distinct from the broad involvement of conventional DCs in coordinating the adaptive response to a broad range of microbes, plasmacytoid dendritic cells (pDCs) are generally considered to be specialized for antiviral immunity (Martin-Moreno and Munoz-Fernandez, 2019). In this context, it is noteworthy that the gonococci unexpectedly elicit a potent pDC response, leading to a robust expression of interferon-α (IFNα), sufficient to cause a profound inhibition of HIV-1 replication in *ex vivo* cultures of peripheral blood cells from HIV-infected patients (Dobson-Belaire et al., 2010). Curiously, this effect is a consequence of the tendency of gonococci to liberate their genomic DNA, either by cellular lysis or their type 4 secretion system, which is detected by TLR9. At first glance, this would seem to contradict the HIV-1 stimulatory effect that has been ascribed to *N. gonorrhoeae*, however pDCs are a double-edged sword with respect to HIV-1 because the IFNα response can independently upregulate expression of viral-specific cellular defenses and recruit activated immune cells that can be infected by the virus (Martin-Moreno and Munoz-Fernandez, 2019). The consequences of this effect remain difficult to surmise without purposeful mucosal sampling of co-infected patients.

In contrast to DCs, tissue resident macrophages are an important latent reservoir of HIV-1 (Meltzer et al., 1990; Clayton et al., 2017). While macrophages are typically infected following CD4 and CCR5 co-receptor engagement, CD4+ T cells remain the preferred HIV-1 target because macrophage display much lower CD4 on their surface. Viral variants that effectively target macrophage have evolved Env protein variants that bind CD4 with much higher affinity, which compensates for its low surface density (Arrildt et al., 2015). A recent study observed that macrophages resident within the human penile urethra can harbor integrated HIV-1, and that these produce infectious virions upon exposure to the TLR4 agonist lipopolysaccharide (Ganor et al., 2019). In the context of male urethral infection by *N. gonorrhoeae*, viral shedding could then be stimulated either directly by macrophage exposure to the gonococci or their liberated products, or indirectly through the exuberant inflammatory cytokine response that is typical of gonorrhea. While yet undescribed, it seems reasonable to assume that a similar effect could occur within the female genital tract.

Beyond the stable effects of *N. gonorrhoeae* on viral replication, it is important to consider that ongoing phase variation might also cause different effects depending upon the virulence factors expressed. Notable in this regard, lipooligosaccharide (LOS) surface structures displaying terminal *N*-acetylglucosamine or *N*-acetylgalactosamine residues are detected by the C-type lectins DC-SIGN on DCs and by MGL on macrophage, respectively. These immune modulatory receptors have the potential to alter the phagocytic responses to other activating stimuli. Consistent with this, gonococcal isolates expressing different LOS structures were found to elicit modest but significant differences in the pattern of cytokines expressed upon *in vitro* infection of these phagocytes, leading to speculation that the adaptive response may skew toward a Th1, Th2, or Th17 bias depending on what phase variable glycan structures are present (van Vliet et al., 2009). As discussed above, phase variable Opa protein-dependent binding to CEACAM1 expressed by DCs can also effectively suppress their maturation in response to activating stimuli including gonococcal infection, preventing the cells from stimulating both CD4+ T cell and CD8+ T cell responses (Yu et al., 2013). It is enticing to think that these effects may have contributed to the observed Th17 immunological bias that suppresses a protective memory response in the mouse lower genital tract infection studies described above. However, whether these differences actually influence the global immune response to *N. gonorrhoeae* and/or HIV-1 during mucosal infection remains to be explored.

The Confounding Effect of Antimicrobial Peptides

It seems intuitive that HIV-1 may benefit from the gonococcal effects aimed at actively suppressing or misguiding otherwise effective immune defenses, or from stimulating an inflammatory response that drives viral expression. However, *N. gonorrhoeae* also triggers the release of neutrophil and epithelial-derived antimicrobial peptides during female genital infection (Wiesenfeld et al., 2002) and gonococcal urethritis in men (Porter et al., 2005). In fact, increased recovery of neutrophil-derived peptides in vaginal swabs is a highly sensitive marker for endometritis among women infected with *N. gonorrhoeae* (Wiesenfeld et al., 2002). While the gonococci can resist most cationic peptides through a combination of lipid A modification and/or efflux pump expression (Kandler et al., 2016), their effect on HIV-1 susceptibility *in vivo* is not so clear. For example, while HIV-1 tends to be susceptible to many antimicrobial peptides *in vitro*, either directly or via activation of innate cellular responses (Klotman and Chang, 2006; Furci et al., 2007), but α-defensins 5 and 6 have been shown to enhance the infectivity of CCR5-tropic HIV-1 by promoting viral aggregation and attachment to the target cell surface (Klotman et al., 2008; Rapista et al., 2011). Notably, many antimicrobial peptides are secreted in a propeptide form that must be processed by neutrophil-released proteases (Porter et al., 2005). Therefore, the relative expression of different peptides and local density of neutrophils, which is obviously high during gonococcal-induced inflammation, is a delicate balance that in theory could combine to either facilitate or inhibit HIV-1 infection. Unfortunately, it seems that the balance is tilted toward increased HIV susceptibility: despite the fact that increased genital levels of α-defensins and cathelicidins (LL-37) were associated with an increased ability of genital secretions to neutralize HIV *ex vivo,* these increases were also associated with increased subsequent

HIV acquisition in both women (Levinson et al., 2009) and men (Hirbod et al., 2014).

GONOCOCCAL EFFECTS ON THE HIV-1 SPECIFIC ADAPTIVE RESPONSE

In women, cervicitis is evident as a purulent discharge consisting almost entirely of neutrophils. While the numbers are smaller, it is notable that there is a significant increase in endocervical CD4+, CD8+, and γδ T cells during uncomplicated gonococcal infection regardless of whether cervicitis is apparent or not (Levine et al., 1998). These effects are a highly localized in that there is no difference in the number of leukocytes in ectocervical or vaginal specimens taken from women with or without *N. gonorrhoeae* infection (Levine et al., 1998), and they do not result from a global increase in systemic lymphocyte counts, which don't significantly change during gonococcal infection (Kaul et al., 2002). It remains unclear whether the apparently targeted recruitment of leukocytes reflects differences in gonococcal attachment or penetration through the epithelial barrier at the endocervix, or whether there are distinct inflammatory responses by epithelia along the genital tract. Regardless, considering that the endocervix is considered to be the prime site for infection with HIV-1, this suggests an obvious site for direct and indirect interactions to occur.

Despite the localized nature of uncomplicated gonococcal infection, there are systemic effects of gonorrhea. Most notably, HIV-1 viremia increases during gonococcal cervicitis (Anzala et al., 2000). While the number of T cells does not change in blood, functional studies suggest that a smaller proportion of HIV epitope-specific CD8+ T cells express IFNγ during gonococcal infection than do after the bacterial infection is treated (Kaul et al., 2002). This appears to be a global effect as a similar reduction in responses to CMV epitopes was also apparent (Kaul et al., 2002), and may reflect a systemic skewing of the T cell response rather than a global suppression of immunity (Anzala et al., 2000). The effect of gonococcal infection is transient in that any effects on lymphocyte response, systemic HIV-1 load or localized viral shedding abate once the bacterial infection is cleared.

Given the chronic nature of HIV-1 infection, clinical studies understandably tend to explore the interactions between *N. gonorrhoeae* and the virus by monitoring changes (or lack thereof) when HIV-infected individuals acquire a gonococcal infection. A rare opportunity to describe the effects of gonococcal infection on HIV-specific immune responses occurred during a large HIV-1 prevention trial involving high risk HIV-negative female sex workers in Nairobi, Kenya, since it involved monthly screening for STIs, including gonorrhea (Sheung et al., 2008). Thirty five of 466 participants acquired HIV-1 during the course of this trial. Quite unexpectedly, the intensity and breadth of HIV-specific CD8 T-cell responses were significantly higher in female sex workers who acquired HIV-1 during a period in which they had a gonococcal infection relative to that apparent in women who were not co-infected (Sheung et al., 2008). This difference was not simply due to increased viral shedding by the transmitting male partner, since *Chlamydia trachomatis* and *Trichomonas vaginalis* also cause urethritis in men but coincident infection with these did not have a similar immune-enhancing effect. Given that gonococcal co-infection did not appear to influence the viral load setpoint once infection is established (Sheung et al., 2008), and that individuals within this cohort have a 5-fold increased risk of acquiring HIV-1 (Kaul et al., 2004), the increased CD8 response was not protective. Instead, it seems reasonable to consider that the increased adaptive response may stem from a combination of higher exposure from the gonococcal-infected seminal fluid and an increased susceptibility of the female partner, perhaps due to the gonococcal-dependent recruitment of target cells into the genital tract or disruption of the epithelial barrier, leading to a higher acute exposure to the virus. In this case, the increased susceptibility and increased immune response may effectively offset each other.

Modeling the Mucosal Response

The relationship between *N. gonorrhoeae* and HIV-1 are complex, and the observational nature of clinical studies prevents the assignment of causality to any effects seen. Models intended to understand molecular and immunologic interactions between the pathogens are difficult to establish, since both *N. gonorrhoeae* and HIV-1 are human-restricted pathogens. Indeed, gorilla, orangutan and chimpanzees may be the only hosts that these two pathogens might naturally co-infect (McGee et al., 1990; Gray-Owen and Schryvers, 1993), and these are neither financially or (more importantly), ethically reasonable models. However, the recent advent of highly elegant mouse models for the study of HIV-1 provides an opportunity to explore some aspects of co-infection. In particular, the engraftment of human $CD34^{+}$ fetal liver or umbilical cord-derived blood stem cells into highly immunodeficient NSG (NOD/*Ltsz-scid*/*scid*γc^{null}) mice allows reconstitution of a leukocyte pool that is human-derived, allowing chronic HIV-1 infection of the human CD4+ T cells (Denton and Garcia, 2011). When these mice were vaginally challenged with *N. gonorrhoeae*, they displayed increased shedding of HIV-1 in genital secretions (Xu et al., 2018). This response occurred without a concomitant increase in systemic viremia, suggesting that the gonococcal infection was stimulating local HIV-1 production within cells of the genital tract. While this model is not trivial to establish, it provides a tractable system to gain mechanistic insight and explore novel therapeutic interventions.

CONCLUSION

Sexually transmitted infections have been referred to as a hidden epidemic (Institute of Medicine, Committee on Prevention and Control of Sexually Transmitted Diseases, 1997; Cohen, 2012), both because they are not openly discussed due to stigma, and also because they may be asymptomatic in over 80% of cases and so are infrequently recognized as an STI since a prospective study of women who are at high risk of HIV acquisition found that only 12.3% of women infected with a pathogen known to cause vaginal discharge had any signs or symptoms of infection

(Mlisana et al., 2012). Thus, even though these co-infections are currently treatable, they continue to drive HIV transmission while they smolder undetected due to stigma and under-diagnosis. This situation is exacerbated in the case of *N. gonorrhoeae* since the recent emergence of multi-drug resistant isolates may soon reduce our ability to effectively treat the infection (Wi et al., 2017). In the absence of an HIV vaccine on the horizon, it is reasonable to consider that interventions targeting these co-pathogens and other pathogens that facilitate HIV transmission may represent one of the achievable short to medium-term strategies to reduce the spread of HIV-1. Considering the substantial impact that gonorrhea in particular has on HIV transmission within a population, further studies aimed to understand the molecular and immunologic determinants of this interaction will hopefully reveal new prevention avenues. A vaccine that confers sterilizing immunity against *N. gonorrhoeae* is a particularly enticing goal when considering that this bacterium does not live outside of humans, which suggests that it could be eradicated. Despite political and economic barriers to the implementation of interventions against STIs will always be contentious, the potential for a lasting positive global health impact on two major pandemics means that there is no better time than the present to get this work underway.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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The MtrCDE Efflux Pump Contributes to Survival of *Neisseria gonorrhoeae* From Human Neutrophils and Their Antimicrobial Components

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The mucosal inflammatory response to *Neisseria gonorrhoeae* (Gc) is characterized by recruitment of neutrophils to the site of infection. Gc survives exposure to neutrophils by limiting the ability of neutrophils to make antimicrobial products and by expressing factors that defend against these products. The multiple transferable resistance (Mtr) system is a tripartite efflux pump, comprised of the inner membrane MtrD, the periplasmic attachment protein MtrC, and the outer membrane channel MtrE. Gc MtrCDE exports a diverse array of substrates, including certain detergents, dyes, antibiotics, and host-derived antimicrobial peptides. Here we report that MtrCDE contributes to the survival of Gc after exposure to adherent, chemokine-treated primary human neutrophils, specifically in the extracellular milieu. MtrCDE enhanced survival of Gc in neutrophil extracellular traps and in the supernatant from neutrophils that had undergone degranulation (granule exocytosis), a process that releases antimicrobial proteins into the extracellular milieu. The extent of degranulation was unaltered in neutrophils exposed to parental or *mtr* mutant Gc. MtrCDE expression contributed to Gc defense against some neutrophil-derived antimicrobial peptides but not others. These findings demonstrate that the Mtr system contributes to Gc survival after neutrophil challenge, a key feature of the host immune response to acute gonorrhea.

Keywords: ***Neisseria gonorrhoeae*, neutrophil, antimicrobial peptide, efflux pump, granule, neutrophil extracellular trap**

INTRODUCTION

Neisseria gonorrhoeae (Gc) is the cause of the bacterial sexually transmitted infection gonorrhea. Gc is notorious for its prevalence (annual estimates in the United States and worldwide of 820,000 and 78 million cases, respectively), its resistance to numerous antibiotics, its association with infertility and other negative sequelae, and its ability to evade and subvert protective immune responses (Satterwhite et al., 2013; Unemo et al., 2016; Rice et al., 2017; Wi et al., 2017). Gc colonizes the human mucosal epithelium of the urogenital tract, pharynx, rectum, and conjunctiva. Epithelial infection stimulates the release of proinflammatory factors that recruit and activate neutrophils (Stevens and Criss, 2018; Stevens et al., 2018). Neutrophils are terminally differentiated, phagocytic granulocytes that make and release cationic antimicrobial peptides and antimicrobial proteins, as well as reactive oxygen species. These antimicrobial products are directed into nascent phagosomes

to kill internalized microbes, or released into the extracellular milieu via granule exocytosis (degranulation) or in DNA-based neutrophil extracellular traps (NETs) to combat extracellular microbes (Nauseef and Borregaard, 2014). Although Gc induces neutrophil degranulation and NET release and is phagocytosed by neutrophils, these activities are not sufficient to clear Gc, and a subset of bacteria survive. Gc resists killing by neutrophils by limiting the release of antimicrobial products by neutrophils and by expressing proteins that defend against those products that the bacteria encounter (Palmer and Criss, 2018). Expression of enzymes that modify lipooligosaccharide, peptidoglycan turnover machinery, a NET-degrading nuclease, and zinc transporters have all been implicated in resistance of Gc to killing by neutrophils (Palmer and Criss, 2018).

The gonococcal multiple transferable resistance (Mtr) efflux pump exports a wide variety of structurally diverse antimicrobial agents, including cationic antimicrobial peptides, antibiotics, fatty acids, non-ionic detergents, and bile salts (Hagman et al., 1995, 1997; Shafer et al., 1998). MtrCDE is a member of the hydrophobic and amphiphilic efflux resistance-nodulation-division family of efflux pumps, which includes *Escherichia coli* AcrAB-TolC and *Pseudomonas aeruginosa* MexAB-OprM (Du et al., 2018). It is composed of inner membrane (MtrD) and outer membrane channels (MtrE), which are connected through a periplasmic membrane fusion lipoprotein (MtrC) (Hagman et al., 1995, 1997;Delahay et al., 1997). Efflux is dependent on energy supplied by the proton motive force that is transduced by MtrD (Janganan et al., 2013; Bolla et al., 2014). In addition to MtrC and MtrD, the MtrE outer membrane channel couples to other efflux pumps in Gc, including FarAB (fatty acid efflux) and MacAB (macrolide efflux), and cooperates with the MtrF inner membrane transporter (sulfonamide efflux) (Lee and Shafer, 1999; Veal and Shafer, 2003; Rouquette-Loughlin et al., 2005). *mtrC, mtrD,* and *mtrE* are found in an operon with a single promoter (Hagman et al., 1995). Expression of *mtrCDE* is directly regulated by a TetR family repressor, MtrR, and an AraC family activator, MtrA (Pan and Spratt, 1994; Hagman and Shafer, 1995; Rouquette et al., 1999). Clinically relevant mutations causing overexpression of MtrCDE can occur in the pump repressor (*mtrR*) or in the promoter region of the *mtrCDE* operon (Zarantonelli et al., 1999; Warner et al., 2008; Ohneck et al., 2011). These mutations confer increased resistance of Gc to antibiotics including penicillin, erythromycin, rifampin, and azithromycin (Hagman et al., 1995; Zarantonelli et al., 1999; Warner et al., 2008; Ohneck et al., 2011). Mutation of *mtrR* is a prerequisite for Gc to acquire porin IB variants (*penB*) that confer high-level resistance to penicillins and cephalosporins (Sparling et al., 1975; Veal et al., 2002; Olesky et al., 2006).

Generally, acquisition of antibiotic resistance alleles is associated with a fitness cost in bacteria. However, clinical and experimental evidence suggests the opposite is true for MtrCDE. Jerse et al. (2003) found that mutations in *mtrCDE* reduced Gc survival in the female murine genital tract, while mutations causing derepression of the *mtrCDE* operon that increase MtrCDE expression enhanced Gc survival (Warner et al., 2007, 2008). MtrCDE may contribute to Gc survival *in vivo* by protecting Gc from the antimicrobial effects of fatty acids and cationic antimicrobial peptides, such as cathelicidins (human LL-37 and mouse CRAMP-38) and other antimicrobials found at inflamed mucosal surfaces (Shafer et al., 1998; Warner et al., 2008). In agreement with this possibility, *mtrCDE* is expressed by Gc in the human urogenital tract of both men and women (McClure et al., 2015; Nudel et al., 2018).

Although MtrCDE is important for Gc infectivity *in vivo* and MtrCDE can efflux a variety of antimicrobials, including host-derived ones, the cellular contexts in which the MtrCDE system contributes to Gc pathogenesis are poorly understood. In this study, we tested the hypothesis that the MtrCDE efflux pump helps defend Gc from killing by neutrophils and their cationic antimicrobial proteins and peptides. In support of this hypothesis, expression of MtrCDE increased Gc viability after exposure to adherent, chemokine-treated primary human neutrophils. MtrCDE specifically enhanced extracellular survival of Gc in NETs, as well as associated with the neutrophil surface. The presence of MtrCDE variably affected resistance of Gc to antimicrobial peptides and proteins made by neutrophils. These findings reveal new roles for MtrCDE during Gc infection.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Piliated, opacity protein (Opa)-deficient Gc of strain FA1090 served as the parent for this study (Ball and Criss, 2013). Gc was maintained on gonococcal medium base (BD Difco) with Kellogg's supplements I + II (GCB) at 37°C, 5% CO_2 (Kellogg et al., 1963). For neutrophil and antimicrobial protein survival experiments, Gc was grown and diluted in rich liquid medium (GCBL) containing Kellogg's supplements and 0.042% sodium bicarbonate for multiple rounds of culture in order to enrich for predominantly mid-logarithmic phase bacteria (Criss et al., 2009).

Construction of *mtr* Mutant and Complement Strains

To generate the *mtrE* mutant, the genomic region surrounding the *mtrE::kan* mutation (RD1) in strain FA19 (from Dr. William Shafer, Emory University) was amplified by PCR using the primer pair MTREF (5′-CGAAGACCAAGGCTTCGTTATGG-3′) and MTRER (5′-AATATTCAATGCCGACCGGACC-3′). The amplicon was introduced by natural transformation into FA1090 parent Gc, and transformants were selected on GCB containing 40 μg/ml kanamycin. Due to issues with PCR amplicon-mediated transformation at the time of strain construction, the *mtrC* and *mtrD* mutants in FA1090 parent Gc were generated by transformation and backcross with genomic DNA from FA19 *mtrC*::*kan* (KH12) or FA19 *mtrD*::*kan* (KH14) (from Dr. William Shafer, Emory University). Genomic DNA was introduced into FA1090 parent Gc by natural transformation, and transformants were selected on GCB containing 40 μg/ml kanamycin. Genomic DNA from one kanamycin-resistant transformant was isolated, verified to have a disrupted *mtr* allele by PCR (see below), and retransformed into the FA1090 parent Gc strain. This procedure was repeated one additional

time, resulting in three consecutive backcrosses in total. Successful replacement of each of the wild-type Mtr genes with its mutated allele was confirmed by DNA sequencing of PCR amplicons that were generated using the following primer sets (located upstream of the *kan* insertion site for each allele): *mtrC,* MTRCF (5′-AGCCTTATCAGGAATGACTGG-3′) and MTRCR (5′-CCATAACGAAGCCTTGGTCTTCG-3′); *mtrD,* MTRDF (5′-CATTGGCAGTGTCGTCTTGC-3′) and MTRDR (5′-CTGCTGCAACAGAGGTCAAGG-3′). Sequencing primers were as follows: *mtrC,* MTRCSEQF (5′-TGCAACCCGTTCGAACATTCG-3′); *mtrD,* MTRDSEQF (5′-AACGGCGTGGAAGGTTTGG-3′); and *mtrE,* MTRESEQF (5′-TTGACCTCTGTTGCAGCAGC-3′). The FA1090 *mtrC* and *mtrD* mutants retained the 1-81-S2 *pilE* sequence, as shown by DNA sequencing of the *pilE* gene that was amplified by PCR using the primer pair PILRBS (5′-GGCTTTCCCCTTTCAATTAGGAG-3′) and SP3A (5′-CCGGAACGGACGACCCCG-3′), with PILRBS serving as sequencing primer. We verified that backcrossing did not result in Opa expression in the FA1090 *mtrC* and *mtrD* mutants, as determined by immunoblotting of bacterial lysates with the 4B12 pan-Opa monoclonal antibody.

To complement the FA1090 *mtrE*::*kan* mutant, Gc was transformed with the pKH35 complementation plasmid (Hamilton et al., 2005) (from Dr. Joseph Dillard, University of Wisconsin, Madison) containing an IPTG-inducible *mtrE* gene. The inducible allele was used in order to be able to titrate MtrE expression. *mtrE* was amplified from the genomic DNA of FA1090 parent Gc using the primer pair: MTRE_KpnI_COMPF (5′-TGC AGG TAC CGC AAA ATA CCG TCT GAG AAC C-3′) and MTRE_SpeI_COMPR (5′-CAG GAC TAG TCG GTT ATT TGC CGG TTT GG-3′). pKH35 and the PCR amplicon were digested with restriction enzymes KpnI and SpeI (New England Biolabs) and ligated together using T4 DNA ligase (New England Biolabs). Transformants were selected on 0.5 μg/ml chloramphenicol. Successful transformants were confirmed by DNA sequencing of a PCR amplicon corresponding to the intergenic *lctP-aspC* site in the Gc chromosome, using the primer pair aspC1 (5′-GCC GGA TGC GTC TTT GTA C-3′) and lctP (5′-GCG CGA TCG GTG CGT TCT-3′). MtrE expression was induced in the complement by adding 250 μg/ml IPTG into GCBL rich liquid medium for 2.5 h before experimental use. At this IPTG concentration, the *mtrE* complement showed statistically indistinguishable sensitivity to the FA1090 parent when challenged with the known MtrCDE efflux pump substrates Triton X-100 (**Figure 1A**) and LL-37 (**Figure 5A**). The *mtrE* complement is designated as *mtrE*+.

Neutrophil Isolation

Neutrophils were purified from the heparinized venous blood of healthy human subjects using dextran sedimentation followed by separation on a Ficoll gradient and hypotonic lysis of erythrocytes, as previously described (Stohl et al., 2005). Neutrophils were resuspended in Dulbecco's phosphate buffered saline (PBS; without calcium and magnesium; Thermo Scientific) containing 0.1% dextrose. Neutrophils were stored on ice for no longer than 2 h before use. All human subjects gave written

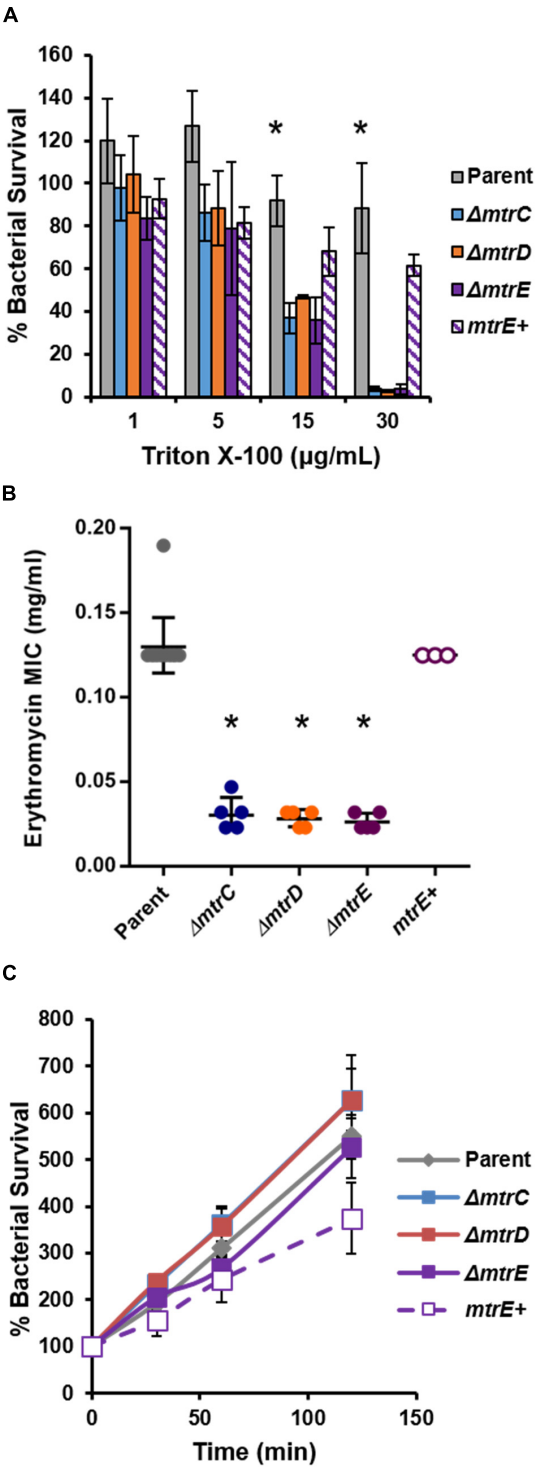

FIGURE 1 | Characterization of MtrCDE efflux pump mutants in piliated, opacity protein-deficient Gc of strain FA1090. **(A)** "Opaless" parent, Δ*mtrC,* Δ*mtrD,* Δ*mtrE*, and *mtrE* complement (*mtrE*$^+$) Gc were exposed to increasing concentrations of Triton X-100 or vehicle control for 1 h. Bacterial survival was calculated as the CFU enumerated after 1 h divided by CFU at the time of inoculation, expressed relative to survival in the vehicle control (100%). Data are presented as the mean ± SEM for 3–4 biological replicates. *$P \leq 0.05$ for

(Continued)

FIGURE 1 | Continued
parent *vs.* each *mtr* mutant at the indicated concentration (one-way ANOVA followed by Tukey's multiple comparisons test). **(B)** Minimal inhibitory concentrations (MIC) for erythromycin were calculated for parent, Δ*mtrC,* Δ*mtrD,* Δ*mtrE*, and *mtrE*$^+$ Gc. Data are presented as the geometric mean MIC ± SD for 3–11 biological replicates. *$P < 0.0001$ for parent vs. each *mtr* mutant and for Δ*mtrE* vs. *mtrE*$^+$ by one-way ANOVA followed by Tukey's *post hoc* test. **(C)** Parent, Δ*mtrC,* Δ*mtrD,* Δ*mtrE*, and *mtrE*$^+$ Gc were inoculated into RPMI containing 10% FBS. Bacterial growth was calculated as the CFU enumerated at each time point divided by CFU at the time of inoculation (0 min). Data are presented as the mean ± SEM for 5–11 biological replicates.

informed consent in accordance with a protocol approved by the University of Virginia Institutional Review Board for Health Sciences Research and the Helsinki Declarations (IRB-HSR protocol #13909).

Bacterial Growth in Rich Liquid Media

Parent, *mtrC* mutant, *mtrD* mutant, *mtrE* mutant, and *mtrE*+ complement Gc were grown in GCBL with $NaHCO_3$ as described above. Mid-logarithmic phase Gc was inoculated into RPMI (Mediatech) with 10% fetal bovine serum (FBS, heat-inactivated; Thermo Scientific) at 1×10^6 colony-forming units (CFU)/ml final concentration in replicate wells of a 24-well plate. Gc was incubated at 37°C, 5% CO_2. At the start of the experiment (time = 0 min) and indicated times thereafter, well contents were mixed thoroughly, and an aliquot from each well was serially diluted and plated on GCB agar. Bacterial growth at each time point is reported relative to CFU enumerated at 0 min for each strain, which is set to 100%.

Bacterial Survival From Human Neutrophils

Neutrophils (10^6 cells per coverslip) were resuspended in RPMI containing 10% FBS and 10 nM interleukin-8 (IL-8, carrier free; R&D Systems). Neutrophils were added to tissue culture-treated plastic coverslips (Sarstedt) in 24-well plates and allowed to adhere for 1 h at 37°C in 5% CO_2. Neutrophils were then challenged with parent, *mtrC* mutant, *mtrD* mutant, *mtrE* mutant, or *mtrE*+ complemented mid-logarithmic phase Gc at a multiplicity of infection of 1 as described previously (Criss et al., 2009). At indicated time points, neutrophils were lysed in 1% saponin, and lysates were diluted and plated on GCB agar. CFU were enumerated from lysates after 20–24 h growth, and percent survival was calculated relative to the CFU enumerated at the start of the experiment (time = 0 min, set to 100%).

Intracellular and Extracellular Bacterial Viability After Neutrophil Challenge

Baclight viability dyes (Invitrogen) were used in conjunction with soybean lectin-Alexa Fluor 647 conjugate (Life Technologies) to discriminate intracellular and extracellular Gc in association with neutrophils after 1 hr of infection as described previously (Johnson and Criss, 2013a).

Bacterial Survival From NETs

Neutrophils (10^6 cells per coverslip) in phenol red-free RPMI (Mediatech) with 5% FBS were treated with phorbol myristate acetate (PMA, 10 nM; Sigma), and allowed to adhere to tissue culture-treated plastic coverslips for 30 min at 37°C, 5% CO_2. Neutrophils were treated with 10 μg/ml cytochalasin D (Sigma) in the presence or absence of 1 U/ml DNase I (New England Biolabs) for 20 min at 37°C, 5% CO_2. Neutrophils were exposed to mid-logarithmic phase Gc at an MOI = 1 and incubated for 1 h at 37°C, 5% CO_2. Percent survival was calculated as the CFU enumerated after 1 hr of neutrophil exposure divided by the CFU added at 0 min.

Neutrophil Degranulation by Flow Cytometry

Surface presentation of granule-specific markers was measured by flow cytometry essentially as described in Ragland et al. (2017). Briefly, adherent neutrophils exposed to Gc were lifted with 5 mM EDTA, washed with DPBS containing 0.1% dextrose, and simultaneously stained with PE-CD63 (Biolegend) and APC-CD66b (Biolegend) as indicators of primary and secondary granule exocytosis, respectively, or respective isotype controls (Biolegend PE-IgG1, κ and Biolegend APC-IgM, κ). Data were acquired using a FACSCalibur Benchtop Analyzer and analyzed using FlowJo software. The geometric means of fluorescence intensity for PE and APC were calculated from a gate that includes all granulocytes by side scatter and forward scatter.

Bacterial Survival After Exposure to Antimicrobial Components

Mid-logarithmic phase Gc was incubated with the antimicrobial component as described below at 37°C, 5% CO_2. CFU were enumerated at 0 min and after incubation for the indicated time. Percent survival was calculated as $CFU_{afterincubation} \div CFU_{0\ min}$ for each concentration of antimicrobial component and expressed relative to Gc survival in the vehicle control, which was standardized to 100%.

Triton X-100

Triton X-100 (Sigma) was diluted in ddH_2O. Gc was incubated in Triton X-100 in 0.5x GCBL for 1 h.

Supernatant From Degranulated Neutrophils

Adherent neutrophils (10^6 cells per coverslip) were treated with PMA (10 nM; Sigma) in RPMI for 30 min at 37°C in 5% CO_2. The supernatant was filtered (0.2 μm) to remove neutrophils. Gc (10^6 CFU) was incubated in 0.2 ml supernatant or RPMI media control for 1 h.

LL-37 Cathelicidin

LL-37 was provided by Dr. William Shafer, Emory University and was diluted in ddH_2O. Gc was incubated in LL-37 in 0.2x GCBL for 1 h.

Bactericidal Permeability-Increasing Protein

BPI (Novateinbio) was diluted in ddH_2O. Gc was incubated with BPI in 0.5x GCBL for 2 h.

Lysozyme

Human lysozyme (Sigma) was diluted in ddH_2O. Gc was incubated with lysozyme in 0.5x GCBL for 3 h.

Azurocidin

Azurocidin (Sigma) was diluted in ddH_2O. Gc was incubated in azurocidin in 0.5x GCBL for 45 min.

Antibiotic Minimal Inhibitory Concentration

Gc susceptibility to erythromycin and vancomycin were measured using *E*-test strips (Biomérieux) as in Ragland et al. (2017).

Statistics

Values are the mean ± the standard error of at least three, independent replicates performed on different days, except for MIC, which was measured as the geometric mean. Significance was assessed using Student's *t*-test, or for multiple comparisons, one-way ANOVA followed by Tukey's *post hoc* test. A *P*-value of < 0.05 was considered significant.

RESULTS

Contribution of the MtrCDE Efflux Pump to Gc Survival From Adherent Human Neutrophils

To test the role of the MtrCDE system in Gc defense against human neutrophils and neutrophil-derived antimicrobials, we introduced insertional mutations in *mtrC, mtrD,* and *mtrE* into the "Opaless" constitutively piliated, Opa protein-deficient Gc of strain FA1090 (Ball and Criss, 2013). Opaless Gc was used as the parent for this study because piliated, Opa protein-deficient Gc exhibits enhanced survival in the presence of human neutrophils, and both pili and Opa protein expression vary at high frequency (Criss et al., 2009; Ball and Criss, 2013; Stohl et al., 2013; Rotman and Seifert, 2014). The *mtrE* mutant was complemented with the *mtrE* gene under the control of an isopropyl-β-D-galactosidase (IPTG)-inducible promoter (*mtrE*$^+$), and was introduced into an ectopic locus in the Gc chromosome. We verified the MtrCDE system was functionally inactivated in the *mtrC, mtrD,* and *mtrE* mutants by their increased sensitivity to killing by two MtrCDE efflux pump substrates, Triton X-100 and erythromycin (**Figures 1A,B**). Complementation rescued the susceptibility of the *mtrE* mutant to the two substrates (**Figures 1A,B**). Inactivation of MtrC, D, or E in the Opaless background had no effect on bacterial growth in rich liquid medium (**Figure 1C**).

We evaluated the contribution of MtrCDE to Gc survival from neutrophils, using adherent, interleukin-8 treated primary human neutrophils to approximate the tissue-localized state of neutrophils after migration to sites of infection (Stevens and Criss, 2018). Parent, *mtr* mutant, and *mtrE* complement Gc were incubated with neutrophils, and bacterial CFU from neutrophil lysates were enumerated over time (**Figure 2**). There was a small but statistically significant decrease in recovery of *mtrE* mutant Gc exposed to neutrophils for 60 and 120 min compared with the parent (**Figure 2**, solid purple line). Recovery of the *mtrE* mutant was restored by complementation (**Figure 2**, dotted purple line). In contrast, the *mtrC* and *mtrD* mutants were not significantly different from parent Gc in their recovery from adherent human neutrophils over time (**Figure 2**, blue and red lines).

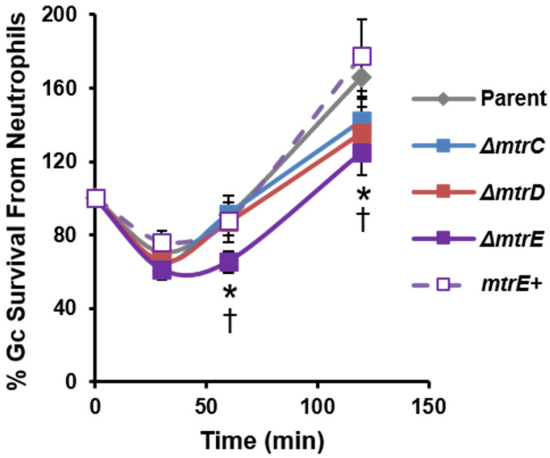

FIGURE 2 | Contribution of the MtrCDE efflux pump to Gc survival from adherent human neutrophils. Parent, Δ*mtrC,* Δ*mtrD,* Δ*mtrE,* and *mtrE* complement (*mtrE+*) Gc were exposed to adherent, IL-8-treated primary human neutrophils for the indicated times. Gc survival was calculated by CFU enumeration at each time point and expressed as the percent of CFU enumerated at 0 min. Data are presented as the mean ± SEM for 5–11 biological replicates. $^*P \leq 0.05$ for Δ*mtrE* vs. parent Gc, and $^\dagger P \leq 0.05$ for Δ*mtrE* vs. *mtrE* complement Gc (Student's two-tailed *t*-test).

The MtrCDE Efflux Pump Enhances Gc Survival From Killing by Neutrophils in the Extracellular Space

The CFU recovery assay used in **Figure 2** reports on the survival of both intracellular and extracellular Gc. In order to directly assess how MtrCDE contributes to Gc survival in each of these locations, we used fluorescent viability dyes alongside a fluorescent lectin to detect extracellular bacteria. Bacteria with permeant membranes stain with the DNA dye propidium iodide, while bacteria with intact membranes exclude this dye and are instead counterstained with SYTO9 (Johnson and Criss, 2013a). In agreement with our previous findings, the viability of Gc was reduced inside neutrophils compared with extracellular, cell surface-associated bacteria (Criss et al., 2009; Johnson and Criss, 2013b). Survival of extracellular *mtrC, mtrD,* and *mtrE* mutant Gc was significantly decreased compared with parent bacteria, and survival of the *mtrE* mutant was rescued by complementation (**Figure 3A**, representative images in **Figure 3C**). In contrast, there were no statistically significant differences in survival of *mtr* mutant Gc inside human neutrophils compared to parent bacteria (**Figure 3A**). There were also no differences in the ability of neutrophils to bind or phagocytose parent, *mtr* mutant, or *mtrE* complement Gc (**Figure 3B**). These results indicate that the

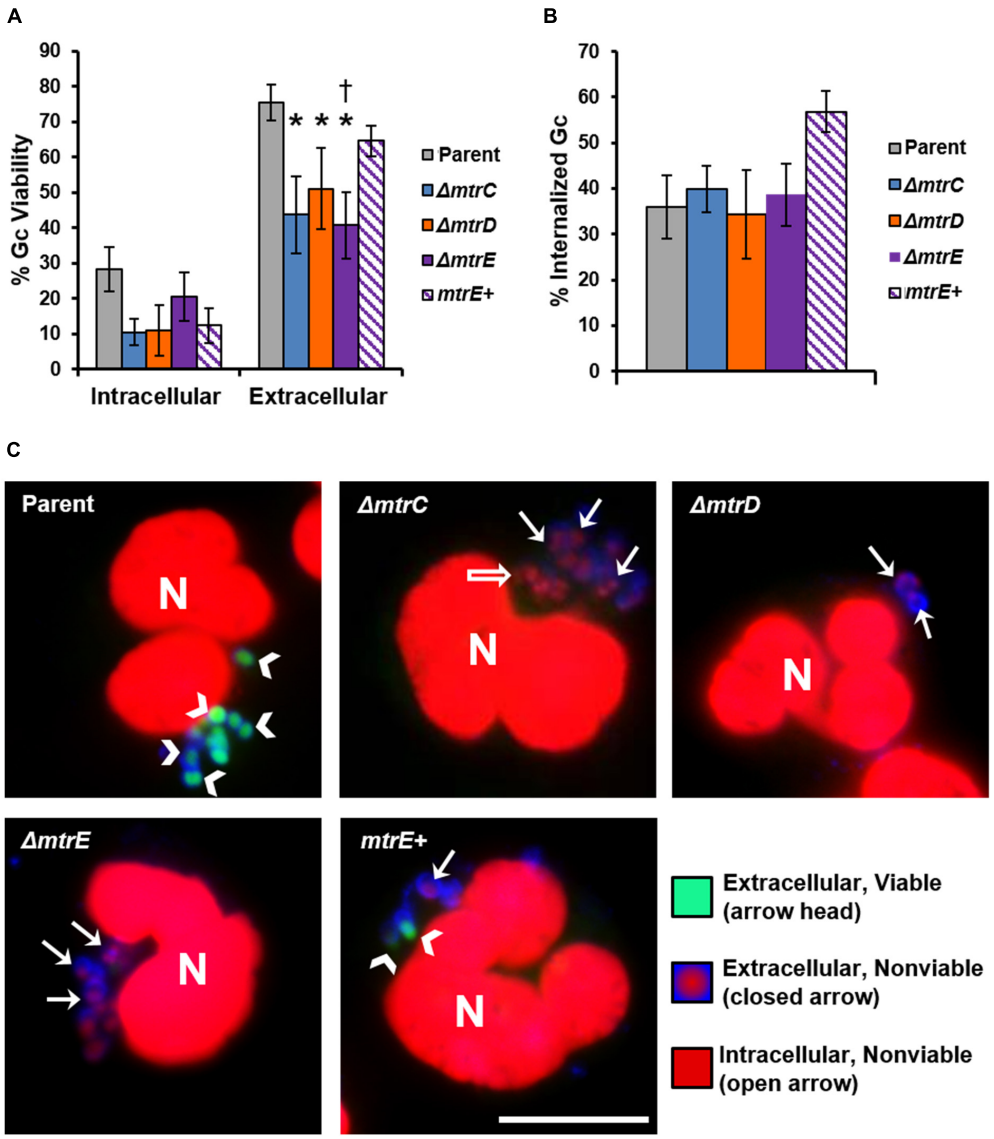

FIGURE 3 | MtrCDE enhances extracellular Gc survival in the presence of adherent human neutrophils. Parent, Δ*mtrC,* Δ*mtrD,* Δ*mtrE*, and *mtrE* complement (*mtrE*+) Gc were exposed to adherent, IL-8-primed primary human neutrophils for 1 h. Extracellular Gc were identified with Alexa Fluor 647-conjugated soy bean lectin (blue), and viable and nonviable Gc were identified with SYTO9 (green) and propidium iodide (red), respectively. **(A)** Percent viable Gc was determined by dividing the number of viable Gc by the total Gc in the intracellular or extracellular compartment for 100–150 neutrophils. Data are presented as the mean ± SEM for 5 biological replicates. $^{*}P \leq 0.05$ for each *mtr* mutant vs. parent, and $^{\dagger}P \leq 0.05$ for Δ*mtrE* vs. *mtrE*+ complement Gc in the extracellular milieu (paired, two-tailed Student's *t*-test). **(B)** The percent of cell-associated Gc that was internalized was calculated by dividing the total number of intracellular Gc by the total number of cell-associated Gc for 100–150 neutrophils. Data are presented as the mean ± SEM for 5 biological replicates. **(C)** Representative fluorescence micrographs per infection condition as indicated in the upper left corner of each image. Arrowheads indicate extracellular viable Gc, closed arrows indicate extracellular nonviable Gc, and open arrows indicate intracellular nonviable Gc. No intracellular viable Gc are apparent in these images. Neutrophil nuclei (N) are also propidium iodide-positive. Scale bar, 10 μm.

MtrCDE efflux pump is specifically important for defending Gc from extracellular killing by neutrophils.

Neutrophils have two modes of extracellular killing: release of antimicrobial agents by granule exocytosis (degranulation) and via NETs. We thus measured survival of Gc after exposure to both extracellular environments. For these and subsequent assays, the *mtrC* mutant was not used since it was expected to phenocopy the *mtrD* mutant. Survival of *mtrD* and *mtrE* mutant Gc was significantly reduced after incubation with supernatant collected from neutrophils that were stimulated to degranulate by treatment with PMA (**Figure 4A**). Survival of the *mtrE* mutant was rescued by complementation (**Figure 4A**). When Gc was exposed to neutrophils that had released NETs after PMA and cytochalasin D treatment (Handing and Criss, 2015), the *mtrE* mutant was significantly reduced in survival compared with the parent and the *mtrE*+ complement (**Figure 4B**). Survival of the

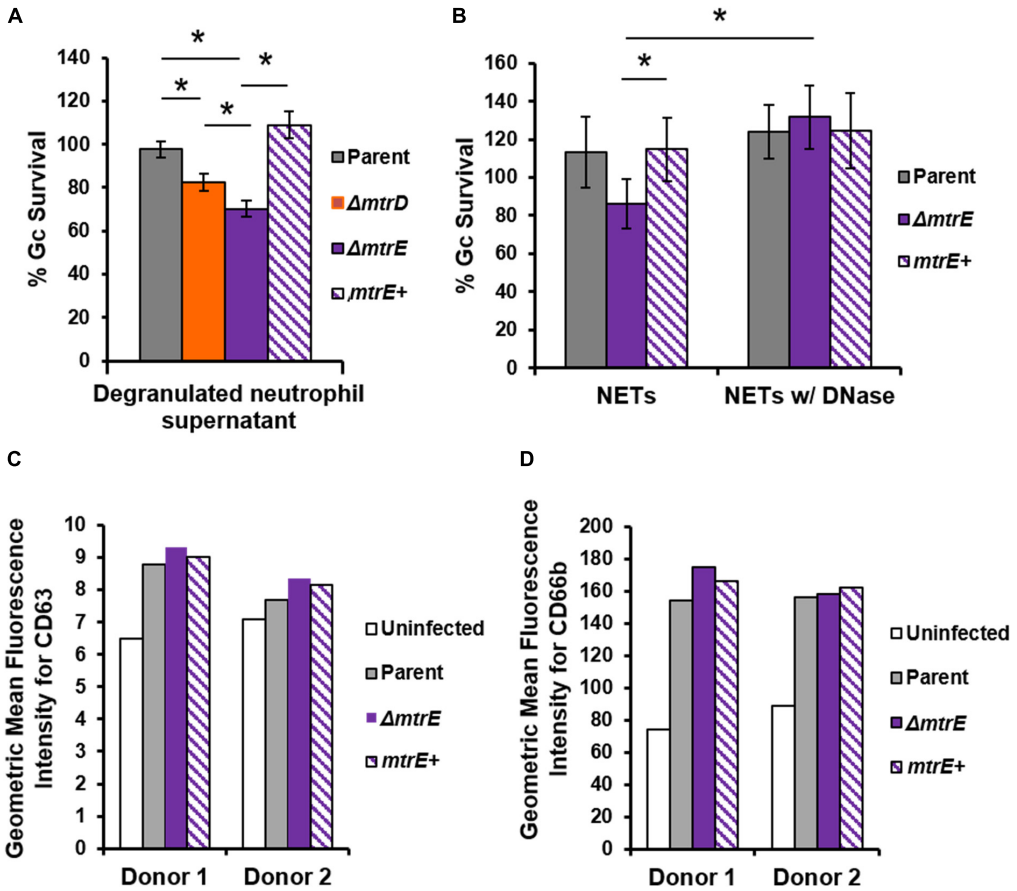

FIGURE 4 | The MtrE outer membrane efflux pump channel contributes to extracellular Gc survival from degranulated neutrophils and from neutrophil extracellular traps. **(A)** Parent, Δ*mtrD*, Δ*mtrE*, and *mtrE* complement (*mtrE*+) Gc were exposed for 1 h to supernatants from PMA-stimulated human neutrophils. Percent Gc survival was calculated as in **Figure 1A**. Data are presented as the mean ± SEM for 3–9 biological replicates. *$P \leq 0.05$ for indicated comparisons (Student's two-tailed *t*-test). **(B)** Parent, Δ*mtrE*, and *mtrE* + Gc were exposed for 1 h to neutrophils treated with PMA and cytochalasin D to produce NETs, in the presence or absence of bovine DNase. Percent Gc survival was calculated by dividing CFU enumerated after 1 h by the inoculum at 0 h. Data are presented as the mean ± SEM for 3 biological replicates. *$P \leq 0.05$ for indicated comparisons (Student's two-tailed *t*-test). The *P*-value for the difference between parent and Δ*mtrE* Gc in the presence of NETs without DNase was 0.06. **(C,D)** Adherent, IL-8 treated human neutrophils were exposed to parent, Δ*mtrE*, or *mtrE*+ Gc for 1 h, or left uninfected in media. Surface exposure of the primary granule component CD63 **(C)** or the secondary granule component CD66b **(D)** was measured by flow cytometry and reported as the geometric mean fluorescence. Results from two independent blood donors are presented.

mtrE mutant was also rescued by addition of exogenous DNase to degrade NETs (**Figure 4B**). There was no difference in the ability of parent or *mtrE* mutant Gc to stimulate neutrophil degranulation, as measured by flow cytometry for surface exposure of primary/azurophilic (CD63) or secondary/specific granule proteins (CD66b) (**Figures 4C,D**). We conclude that the Mtr efflux pump, especially the MtrE channel, enhances the resistance of Gc to extracellular mechanisms of killing used by neutrophils.

The MtrCDE Efflux Pump Defends Gc From Select Antimicrobial Proteins and Peptides Made by Human Neutrophils

Given that Mtr expression helped Gc resist killing by the degranulated supernatant of human neutrophils (**Figure 4A**), we sought to identify neutrophil products that were substrates for efflux by MtrCDE. Parent, *mtrD* mutant, *mtrE* mutant, and *mtrE* complement Gc were incubated with purified neutrophil antimicrobial proteins, and survival was measured by enumerating CFU after incubation (see Materials and Methods). In accordance with previous reports (Shafer et al., 1998), we found that the *mtrD* and *mtrE* mutant in the Opaless background were significantly more sensitive to killing by LL-37 than either parent or *mtrE*+ complement Gc (**Figure 5A**). In contrast, the *mtrE* mutant, but not the *mtrD* mutant, was more sensitive to killing by bactericidal-permeability increasing protein (BPI) (**Figure 5B**). The *mtrD* and *mtrE* mutants showed no difference in survival compared to parent or *mtrE*+ Gc after incubation with human lysozyme (**Figure 5C**).

Unexpectedly, the *mtrE* mutant was more resistant to the primary granule antimicrobial protein azurocidin than the parent, *mtrD* mutant, or *mtrE*+ complement (**Figure 5D**). This finding led us to explore if there were other antimicrobial

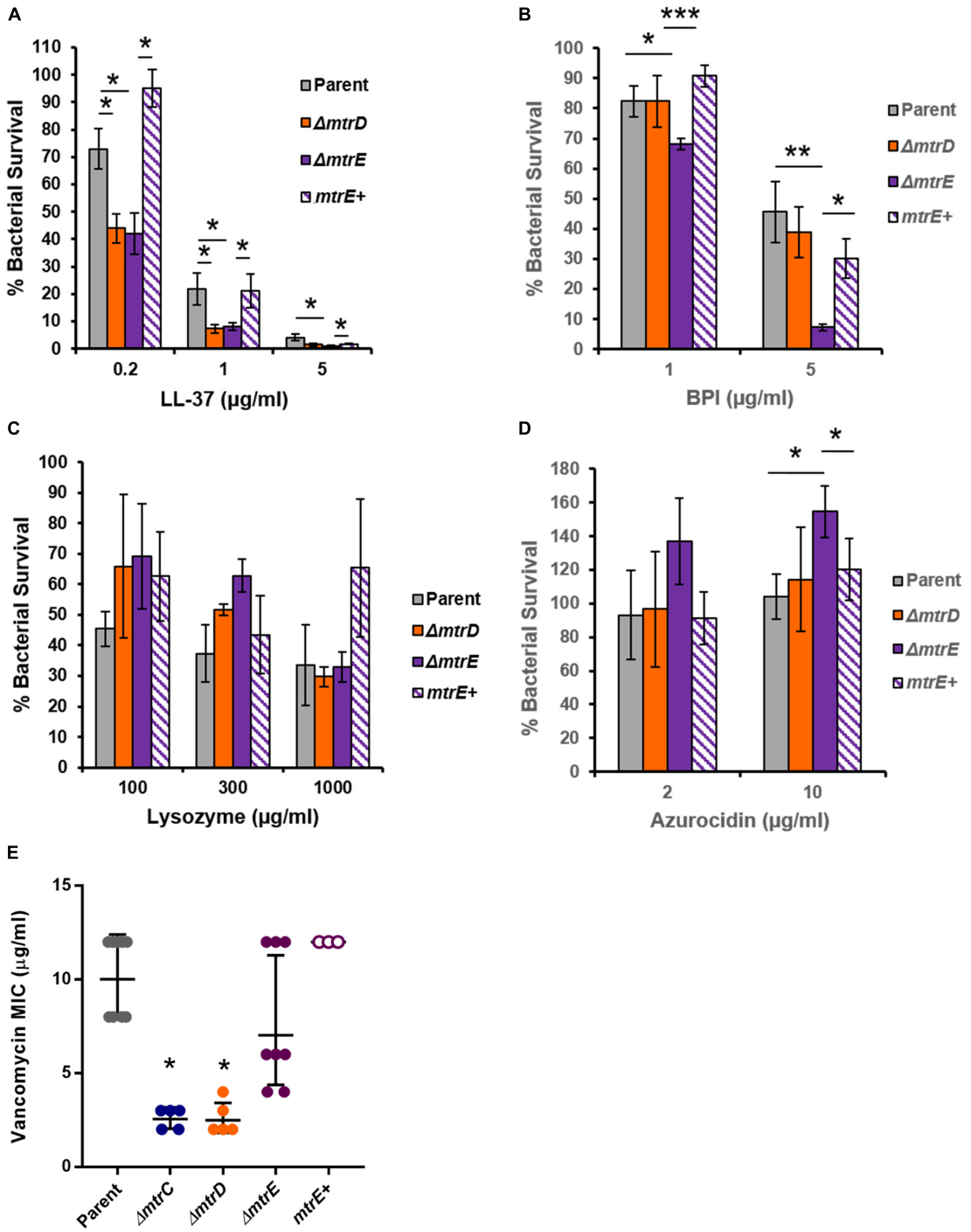

FIGURE 5 | MtrCDE efflux pump components enhance Gc survival in the presence of selected neutrophil granule components. **(A)** Parent, Δ*mtrD*, Δ*mtrE*, and *mtrE* complement (*mtrE*+) Gc were exposed to LL-37 at the indicated final concentrations for 1 hr. Percent Gc survival was determined as in **Figure 1A**. Data are presented as the mean ± SEM for 3–9 biological replicates. $^*P \leq 0.05$ for the indicated comparisons (Student's two-tailed *t*-test). **(B)** Parent, Δ*mtrD*, Δ*mtrE*, and *mtrE*+ Gc were exposed to BPI at the indicated final concentrations for 45 min. Percent Gc survival was determined as in **Figure 1A**. Data are presented as the mean ± SEM for 3–7 biological replicates. $^*P \leq 0.05$, $^{**}P \leq 0.025$, and $^{***}P \leq 0.01$ for the indicated comparisons (Student's two-tailed *t*-test). **(C)** Parent, Δ*mtrD*, Δ*mtrE*, and *mtrE*+ Gc were exposed to lysozyme at the indicated final concentrations for 3 h. Percent Gc survival was determined as in **Figure 1A**. Data are presented as the mean ± SEM for 3–4 biological replicates. **(D)** Parent, Δ*mtrD*, Δ*mtrE*, and *mtrE*+ Gc were exposed to azurocidin at the indicated final concentrations for 45 min. Percent Gc survival was determined as in **Figure 1A**. Data are presented as the mean ± SEM for 3–6 biological replicates. $^*P \leq 0.05$ for the indicated comparisons (Student's two-tailed *t* test). **(E)** MICs for vancomycin were calculated for parent, Δ*mtrC*, Δ*mtrD*, Δ*mtrE*, and $mtrE^+$ Gc. Data are presented as the geometric mean MIC ± SD for 3–9 biological replicates. $^*P < 0.0001$ for Δ*mtrC* vs. parent, Δ*mtrE*, or $mtrE^+$ and Δ*mtrD* vs. parent, Δ*mtrE*, or $mtrE^+$ by one-way ANOVA followed by Tukey's *post hoc* test. Parent, Δ*mtrE*, and $mtrE^+$ Gc were not significantly different from one another.

compounds where the *mtrD* mutant was more susceptible than the *mtrE* mutant. Gram-negative bacteria like Gc are intrinsically resistant to vancomycin, unless their outer membrane is somehow breached (Ragland et al., 2017). The *mtrC* and *mtrD* mutants had a significantly lower MIC to vancomycin compared with parent or *mtrE* mutant Gc (**Figure 5E**). Unlike results with azurocidin, the parent, *mtrE* mutant, and *mtrE*+ complement all had similar sensitivity to vancomycin (**Figure 5E**).

Together, these findings show that the MtrCDE efflux pump and the MtrE outer-membrane channel contribute in different ways to defense against neutrophil-derived antimicrobial proteins and peptides.

DISCUSSION

The gonococcal *mtr* system was first identified 45 years ago (Maness and Sparling, 1973). While many of the diverse substrates subject to efflux by MtrCDE have been identified, when and where Gc employs the MtrCDE system during infection have not been fully resolved. In this study, we showed that the MtrCDE efflux pump contributes to resistance of Gc to killing by human neutrophils. Specifically, MtrCDE enhances Gc survival extracellularly, in NETs and upon exposure to neutrophil products that are released by degranulation. Using purified cationic antimicrobial proteins and peptides made by neutrophils, we identified four phenotypes associated with MtrCDE in Gc: (1) MtrCDE-dependent sensitivity (e.g., LL-37), (2) MtrE-dependent but MtrD-independent sensitivity (e.g., BPI), (3) MtrD-dependent but MtrE-independent sensitivity (e.g., azurocidin, vancomycin), (4) no effect of MtrCDE (e.g., lysozyme). These findings suggest a location- and component-specific defense conferred by the MtrCDE efflux pump when Gc is confronted by neutrophils, as during acute human infection.

MtrCDE improves Gc survival in the murine vaginal tract (Jerse et al., 2003; Warner et al., 2007, 2008), suggesting that the efflux pump may be involved in export of antimicrobials released by host mucosal epithelial cells. We demonstrated that MtrCDE protects Gc from extracellular antimicrobials released from human neutrophils, and protects Gc from killing by NETs that contain various antimicrobial proteins. It is notable that the *mtrC* and *mtrD* mutants showed a survival defect when enumerated by microscopy for bacterial viability, while they were not statistically different from the parent by CFU enumeration. This is in contrast to the *mtrE* mutant, which showed a significant decrease in survival by both assays. The CFU assay accounts for the viability of all bacteria in the infection: intracellular, extracellularly cell-associated, and free in the extracellular milieu. In contrast, free extracellular bacteria are lost when the infected neutrophils are processed for fluorescence microscopy with the viability dyes. The *mtrD* mutant was significantly less susceptible than the *mtrE* mutant to killing by a mix of antimicrobial peptides and proteins that were released from degranulated neutrophils (**Figure 4A**). Thus we anticipate that the decreased viability of only the *mtrE* mutant by the CFU assay is due to this non-cell-associated, extracellular fraction of bacteria.

Although neutrophil phagosomes that have fused with granules contain high concentrations of antimicrobial peptides and proteins, MtrCDE was dispensable for survival of Gc inside neutrophils. One explanation for this surprising observation is that Gc-containing phagosomes do not contain substrates for efflux through MtrCDE. We previously reported that Opa-negative Gc, as used in this study, resides in an immature phagosome that has undergone fusion with secondary but not primary granules (Johnson and Criss, 2013b). Secondary granules contain hCAP18, which is processed by primary granule proteases to generate the mature antimicrobial peptide LL-37 (Sorensen et al., 2001). Thus a primary granule-negative phagosome is not expected to contain mature LL-37. BPI and azurocidin are also predominantly found in primary granules (Nauseef and Borregaard, 2014). However, we did not measure any differences in MtrCDE-dependent Gc survival inside neutrophils when the bacteria were first opsonized with IgG, which directs them into primary granule-positive phagolysosomes (Johnson and Criss, 2013b) (data not shown). Alternatively, antimicrobial proteins could reach a concentration inside the Gc-containing phagosome that overwhelms MtrCDE efflux activity, such that no susceptibility phenotype is observed for *mtr* mutants. However, we do not favor this possibility because we have not measured significant differences in the intracellular viability profile of wild-type Gc of the FA1090 and MS11 backgrounds; FA1090 naturally expresses lower MtrCDE levels compared to MS11 due to natural mutations in *mtrA* and *mtrR,* respectively (Rouquette et al., 1999; Ohneck et al., 2011). Studies to test the role of MtrCDE in strains that more robustly express the efflux pump, to measure *mtr* expression in Gc residing inside neutrophils, and to identify the cohort of antimicrobials inside Gc phagosomes would help to clarify this issue.

NET formation is an important component of the host innate immune response to many bacterial pathogens (Brinkmann et al., 2004). Both Gc and *Neisseria meningitidis* can induce NET release from human neutrophils *in vitro* (Lappann et al., 2013; Gunderson and Seifert, 2015; Juneau et al., 2015). Gc and *N. meningitidis* modify the lipid A portion of their lipooligosaccharide (LOS) with phosphoethanolamine (PEA), which reduces susceptibility to killing by several neutrophil-derived antimicrobial components including LL-37, and protects them from killing within NETs (Lappann et al., 2013; Handing and Criss, 2015; Juneau et al., 2015; Kahler et al., 2018). We have shown here that the MtrCDE efflux pump also contributes to Gc survival in the presence of neutrophils induced to make NETs. In contrast, MtrCDE does not enhance survival of *N. meningitidis* exposed to NETs, which may be due to virulence factors such as the capsule that additionally defend against NETs (Lappann et al., 2013).

Many of the antimicrobial peptides and proteins made by neutrophils and released by degranulation have amphipathic characteristics, which are a feature shared by the structurally diverse substrates of the MtrCDE efflux pump. Amphipathic molecules possess regions of hydrophobic and hydrophilic elements that tend to favor interaction with biological membranes (Wiesner and Vilcinskas, 2010). The LL-37 antimicrobial peptide, which is made by neutrophils as

well as epithelial cells, is an amphipathic molecule and a well-described MtrCDE efflux pump substrate. Many other neutrophil antimicrobial proteins and peptides have amphipathic characteristics, including α-defensins, lysozyme, BPI, CAP37/azurocidin, lactoferrin and cathepsin G, yet not all of these showed MtrCDE-dependent effects on Gc survival. Susceptibility to these antimicrobial peptides and proteins was not correlated with their molecular weight and potential ability to be exported through the 22 Å MtrE pore (Lei et al., 2014). For instance, the *mtrE* mutant did not have increased sensitivity to lysozyme (14.5 kDa), but was more sensitive to BPI (55 kDa). Given that the MtrE outer membrane channel couples to multiple efflux pump systems in Gc (Lee and Shafer, 1999; Veal and Shafer, 2003; Rouquette-Loughlin et al., 2005), these findings either suggest an MtrE-dependent but MtrCD-independent efflux pump exports the antimicrobial proteins, multiple MtrE-dependent efflux pumps work in concert for export, or the presence of MtrE changes the properties of the outer membrane to render it more resistant to antimicrobial protein and peptide attack. Future studies will explore how these and other efflux systems contribute to Gc susceptibility to neutrophil-derived antimicrobials.

One surprising finding arising from this study was that loss of the MtrE outer membrane channel enhanced, not reduced, Gc survival after exposure to azurocidin. Along these lines, loss of MtrE had no effect on sensitivity to vancomycin, but loss of MtrC and MtrD did. In both cases the phenotype of the *mtrE* mutant was rescued by complementation, suggesting the effects were due to MtrE and not a second site mutation. This raises the intriguing possibility that MtrE is exploited by selected antimicrobials as a portal across the outer membrane. This is counterintuitive since efflux pumps should work unidirectionally in export, yet small molecules like antibiotics and heme can transit across the outer membrane through channels such as the PorB porin and the PilQ pilus channel (Chen et al., 2004; Zhao et al., 2005; Olesky et al., 2006; Lindberg et al., 2007). Blocking antibodies, channel inhibitors, or mutations that sterically hinder channel activity are all approaches that can be used to test these possibilities (Janganan et al., 2011, 2013; Wang et al., 2018). It is also possible that loss of *mtrE* has secondary effects on protein or lipooligosaccharide abundance or composition of the outer membrane, to render Gc more resistant to a subset of antimicrobials.

The *mtrCDE-mtrR* locus has been recently described as a hotspot for genetic recombination (Wadsworth et al., 2018), and antibiotic-resistant strains of Gc frequently carry mutations that inactivate MtrR, leading to increased MtrCDE expression (Rice et al., 2017). These variations may translate into overall effects on Gc fitness *in vivo,* as seen in the female murine genital tract and potentially in the human urethral Gc challenge model (Jerse et al., 2003; Warner et al., 2007, 2008; Hobbs et al., 2011). Based on our findings, the antimicrobial activities of neutrophils are yet another stressor with which Gc contends by using the MtrCDE efflux pump. Interestingly, Nudel et al. (2018) recently reported that *mtrCDE* expression was twofold higher in Gc isolated from the male urethra compared with the female genital tract, including in individuals carrying the same bacterial strain. Therefore, we posit that changes in expression of MtrCDE assist Gc in successfully colonizing host sites that differ in neutrophil load and neutrophil activation state, including male vs. female hosts, lower vs. upper genital infection, and genital vs. extragenital sites of infection.

AUTHOR CONTRIBUTIONS

JH, SR, and AC contributed to the conception and design of the study. JH, SR, and UB performed the experiments and analyzed the results. JH, SR, and AC wrote the manuscript. All authors performed statistical analyses and contributed to the editing of the manuscript prior to submission and read and approved the submitted version of the manuscript.

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MINI REVIEW
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Macrophage–*Neisseria gonorrhoeae* Interactions: A Better Understanding of Pathogen Mechanisms of Immunomodulation

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Neisseria gonorrhoeae is a significant health problem worldwide due to multi-drug resistance issues and absence of an effective vaccine. Patients infected with *N. gonorrhoeae* have not shown a better immune response in successive infections. This might be explained by the fact that *N. gonorrhoeae* possesses several mechanisms to evade the innate and adaptative immune responses at different levels. Macrophages are a key cellular component in the innate immune response against microorganisms. The current information suggests that gonococcus can hijack the host response by mechanisms that involve the control of macrophages activity. In this mini review, we intend to condense the recent knowledge on the macrophage–*N. gonorrhoeae* interactions with a focus on strategies developed by gonococcus to evade or to exploit immune response to establish a successful infection. Finally, we discuss the opportunities and challenges of therapeutics for controlling immune manipulation by *N. gonorrhoeae*.

Keywords: innate immunity, macrophages, *Neisseria gonorrhoeae*, host response, immunomodulation

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INTRODUCTION

Neisseria gonorrhoeae or gonococcus, a gram-negative diplococcus, belongs to the genus *Neisseria* and is the etiological agent of the sexually transmitted bacterial infection (STI) gonorrhea. Nowadays, gonorrhea is the second most common bacterial STI and results in substantial morbidity and economic cost worldwide. The majority of infections are benign mucosal infections of the urogenital tract, pharynx, and rectum. Ascended infections such as endometritis, salpingitis, epididymitis, and pelvic inflammatory disease (PID) are more difficult to treat. Gonococcal PID and its related complications (infertility, ectopic pregnancy, and chronic pelvic pain) constitute the major morbidity and mortality associated with gonorrhea (1). The most recent annual incidence estimates, based on data collected in 2012, indicate 78 million new cases worldwide with a global incidence rate of 19 per 1,000 females and 24 per 1,000 males (2, 3). Another serious concern is the current identification of antimicrobial drug resistance of *N. gonorrhoeae*, which includes the most recent fluoroquinolones and extended-spectrum cephalosporin (4–6). Indeed, U.S. Centers for Disease Control (CDC) identified multidrug resistance as among the 3 most "urgent" hazard-level threats to the U.S. population (7).

Recent information shows that *N. gonorrhoeae* can prevent the development of a successful protective immune response; The causes of the weak immune response triggered by *N. gonorrhoeae* are varied and correspond to multiple mechanisms that include the immune privilege of the reproductive tissue colonized by bacteria (8, 9), as well as the own strategies developed by the bacteria, such as epitope mimicry, antigenic variations and phase variation. Moreover, gonococci seem to directly interfere with the cells involved in adaptive immune response, such as dendritic cells and B and T lymphocytes (10–13). Regarding innate immunity, phagocytic cells such as macrophages and neutrophils (PMN) represent the earliest line of defense against invading bacteria. However, PMN cannot clear infections by *N. gonorrhoeae* (14). Since gonococcus can survive into PMN and suppress the oxidative burst (14, 15), it has been hypothesized that bacteria actively recruit PMN to permit the spread to profounder tissues of the host and even to other hosts.

Moreover, *N. gonorrhoeae* also affects macrophages and their functionality, posing additional difficulties to its detection and elimination by the innate immune system (16).

Considering that macrophages are critical cells in the innate immune response to *N. gonorrhoeae*, and macrophage-driven innate immune response can be subverted by *N. gonorrhoeae*, promoting the persistence of gonorrhea, we will highlight our current knowledge about macrophage–*N. gonorrhoeae* interactions in this mini review with a focus on strategies developed by gonococcus to evade or to exploit immune response to establish a successful infection.

MACROPHAGES AND ANTIMICROBIAL RESPONSE

Macrophages are present in almost all tissues and have diverse functions ranged from clearance of microbes, dead, and senescent cells until reparative and regulatory functions. Tissue-resident macrophages can derive from yolk sac macrophages, fetal liver monocytes, or adult bone-marrow monocytes capable of entering in tissues during inflammation (17, 18). When bacteria cross the layer of epithelial cells, accessing submucosa, they have the first encounter with macrophages (19). Macrophages recognize microbial pathogen-associated molecular patterns (PAMPs) and endogenous danger-associated molecular patterns (DAMPs) by genome-encoded pattern recognition receptors (PRRs). It leads the activation of host defense pathways, which include internalization of the pathogen into phagosomes and fusion with lysosomes to form phagolysosomes, where the microbes are killed by reactive oxygen and nitrogen species and proteolytic enzymes, resulting in the clearance of the infection (20). Also, activation of macrophages includes secretion of pro-inflammatory cytokines and antigen-presentation (21). Currently, it has been demonstrated that many signals recognized by macrophages drive to classical activation (M1 macrophages) or alternative activation (M2 macrophages) (22). While M1 macrophages stimulate a robust anti-tumor and microbicide response, M2 macrophages are involved in tumor progression, tissue remodeling, response against parasites and they have regulatory functions (23).

CELLULAR MODEL FOR EXAMINING MACROPHAGES DURING INFECTION

Diverse cell models have been used to study the interaction between *N. gonorrhoeae* and macrophages; these include cell lines and primary macrophages. The most common murine line used is RAW 264.7. This is a tumor line induced by Abelson murine leukemia virus (24). Human macrophage cell lines mostly used include U937 and THP-1. THP-1 cells are derived from acute monocytic leukemia (25) and U-937 cells were obtained from a patient suffering from histiocytic lymphoma (26). We should consider the differences between these lines based on their diverse origin and maturation stage (27).

Moreover, cell lines generally vary from primary macrophages, since repeated subculture typically results in some abnormalities such as the loss of genes expression (28); although this is not important for proliferation, it is critical for the immune function developed by macrophages. Because of these deficiencies of macrophage cell lines, it may be essential to complement such a model with culture of primary macrophages cells. Cell sources for primary macrophage models include mouse peritoneal macrophages (PM), bone marrow-derived macrophages (BMDM) and human monocyte-derived macrophage (MDM). Murine source of macrophages can be obtained from different strains of wild-type, transgenic and gene-targeted mice. Although PM can be easily harvested, the output is small and we should consider that sanitary and welfare conditions of the animal could affect the physiology of macrophages (29).

Conversely, BMDM do not present problems associated with the health status of the donor mice, because BMDM are obtained from bone marrow stem cells and are completely differentiated *in vitro* (29). In this line, human peripheral blood monocytes are the most commonly used precursors for generating macrophages *in vitro*. The MDM culture allows the monocytes differentiation and polarization toward different macrophage phenotypes using cytokines and/or bacterial products. Thus, M1 and M2 macrophages require GM-CSF or M-CSF in the presence of IFN-γ and/or LPS and IL-4 or IL-13, respectively (30, 31).

MECHANISMS OF SURVIVAL AND REPLICATION OF *N. gonorrhoeae* DURING MACROPHAGE INFECTION

Neisseria gonorrhoeae is able to prevent some defense mechanisms elicited by macrophages. Our laboratory and others have showed different mechanisms used by gonococcus to evade antimicrobial macrophage response. Below, we discuss some of the most relevant mechanisms observed in Macrophage–*N. gonorrhoeae* interactions.

MODULATION OF PHAGOCYTOSIS BY MACROPHAGE

Neisseria gonorrhoeae can avoid phagocytosis mediated by opsonic antibodies. The membrane molecules such as lipooligosaccharide (LOS), Pili, Opa, and Porin (Por) are the main targets to generate antibodies against gonococci (32–34). By constantly varying these antigens, the bacteria elude antibody opsonization and successive IgG Fc-receptor-mediated phagocytosis (14, 35, 36). Additionally, LOS carbohydrates moiety mimic human surface antigens (37, 38) impairing the recognition by macrophages. Other membrane components such as pili reduce the association *in vitro* between *N. gonorrhoeae* and non-activated mouse PM macrophages, similar to streptococcal M protein which reduce the phagocytosis (39). However, the rate of phagocytosis of piliated gonococcus can be enhanced using immune anti-serum, indicating that this enhancement reflects primarily immunoglobulin G-mediated phagocytosis (opsonization through Fc receptor) rather than surface attachment (40, 41). In spite of this, later studies showed that pili (particularly pilin subunit PilE) acts as a factor that promotes non-opsonic ingestion of gonococci by human monocytes in absence of serum factors rather than as a protective factor (42).

MODULATION OF INTRACELLULAR KILLING BY MACROPHAGE

It has been demonstrated that human monocytes and macrophages can kill intracellular gonococci (39) with diverse kinetics, ranged from a complete killing after 30 min of incubation in mouse peritoneal macrophages (43) to a more prolonged period of survival in RAW 264.7 cells and human MDM (44). When using membrane-impermeable antibiotics, such as gentamicin, our group and others have seen that the number of intracellular gonococcus inside macrophages increases over time (27, 45, 46). The persistence of *N. gonorrhoeae* inside macrophages is indicative of resistance mechanisms to innate host defenses. Thus, studies in monocytes showed that Opa-positive and piliated gonococci caused a differential oxidative response (42). Château et al. showed in differentiated U937 cells that internalized bacteria could escape from phagosome or endosome and retain viability (27).

Moreover, studies using MDM showed that purified PorB modifies phagosomal processing and reduces the delivery of the lysosomal enzyme cathepsin D, suggesting a delay in phagosome maturation and oxidative killing mechanisms (47), which is consistent with the absence of colocalization of *N. gonorrhoeae* with Lysosomal-associated membrane protein 1(LAMP-1) present in the lysosomal acidic compartment (27). In this way, two other proteins have been described, Ng-MIP (with homology to Macrophage infectivity potentiator), and Ng-OmpA (with homology to the outer membrane protein A). Ng-OmpA, unlike Ng-MIP, has been associated with the adhesion and internalization of *N. gonorrhoeae* in RAW 264.7 cells (44, 48). However, both proteins have shown that they protect bacteria from being killed by macrophages, probably through mechanisms related to the activity of peptidylprolyl cis/trans isomerase (PPIase) (44) and the inhibition of apoptosis of infected macrophages (48), as observed in other pathogens (49–51). Intracellular survival is also favored by the modulation of cellular iron metabolism by gonococcus. Infection of human monocytes, THP-1, MM6, and murine RAW 264.7 cell lines showed an upregulation of hepcidin, neutrophil gelatinase-associated lipocalin (NGAL) and Natural resistance-associated macrophage protein 1 (NRAMP1), suggesting an increase in cellular iron bioavailability (52). Similarly, infection of BMDM with *N. gonorrhoeae* was associated with increased availability of intracellular iron, associated with INF-β and cGAS/STING signaling pathway (53).

MODULATION OF MACROPHAGE CELL DEATH

Apoptosis and autophagy constitute cell death modes for the elimination of infected cells. Failure in these mechanisms can favor the advance of disease (54, 55). *Neisseria gonorrhoeae* infection and its effects on apoptosis have been studied in PMN, and epithelial cells showed different results (56–59). A study using various sources of macrophages showed that gonococcus inhibit apoptosis in U937 and MDM cells in intrinsic and extrinsic pathways, but in THP-1 cells only extrinsic apoptotic pathway was affected (27). Concerning autophagy, Zughaier et al. showed that a phosphoethanolamine (PEA) modification of lipid A increases *N. gonorrhoeae* survival through evasion of autophagy affecting the TLR4-mediated induction of autophagic flux in RAW264 and THP-1 macrophages (60). Additionally, cell death can arise by pyroptosis. Studies using *N. gonorrhoeae* or isolated *N. gonorrhoeae* LOS showed cell death through NLRP3-mediated pyronecrosis in THP-1 cells (61) and cell death in human macrophages by activation of both canonical and non-canonical pyroptosis pathways (62).

MODULATION OF MACROPHAGE ACTIVATION

Several membrane components from *N. gonorrhoeae* including LOS play a role during infection and modulation of antimicrobial response (63, 64). The immunomodulatory potential of LOS is based on lipid A backbone rather than carbohydrates structure. In this context, Patrone et al. showed that the variation in carbohydrates structure from LOS has no influence on production of IL-8, IL-12, and TNF-α cytokines (65). Indeed, THP-1 cells showed differences in their inflammatory response, TLR4/MD2 signaling or TNF-α production, toward LOS derived from a variety of *Neisseria* strains independent of the oligosaccharide truncation (66). Contrarily, the presence of single lauric acid residue in a determinate position of Lipid A and a hexa-acylated lipid A seems to be critical for the initiation of pro-inflammatory responses, since *lpxLII* and *msbB* mutants

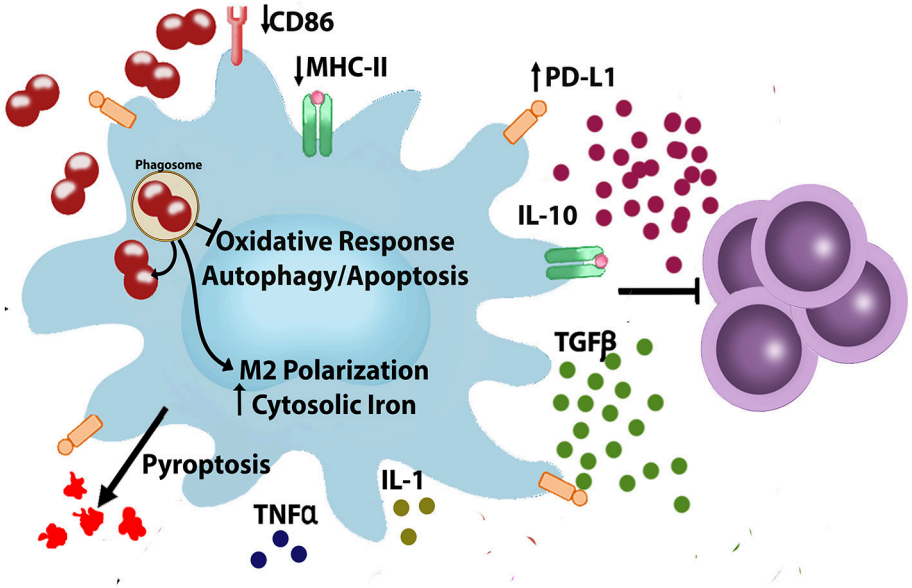

FIGURE 1 | *Neisseria gonorrhoeae* evades and modulates macrophages. During infection, *N. gonorrhoeae* interact with immune cells such as macrophages. In macrophages *N. gonorrhoeae* is able to escape from phagosome (27, 42, 45–48), modulate cellular iron metabolism (52, 53), inhibit apoptosis and autophagy (27, 60), modulate production of inflammatory/anti-inflammatory cytokines (63–71, 83), and polarizes macrophages, resulting in macrophages that are less capable of T cell proliferation (45). CD86, Cluster of Differentiation 86; IL- 1, Interleukin 1; IL-10, Interleukin 10; MHC, Major Histocompatibility Complex; PD-L1, Programmed Death-Ligand 1; TGF-β, Transforming Growth Factor-beta; TNF-α, Tumor Necrosis Factor-alpha.

that lost these features showed a reduced ability to induce pro-inflammatory cytokines and stimulation of TLR-4-inflammatory signaling in U937, THP-1 and mouse primary macrophages (67, 68).

Similarly, modification of gonococcal lipid A with PEA also influences the production of CXCL3 and CXCL10 chemokines in infected macrophages (60). Besides, Knilans et al. showed that other molecules like peptidoglycan monomers released during *N. gonorrhoeae* cell wall remodeling by LtgA and LtgD lytic transglycosylases, also suppress TNF-α and IL-1β production in THP-1 cells by modulation TLR-2 and NOD2 signaling pathways (69). In this way, it is possible to hypothesize that Opa binding to CEACAM1 present on monocytes may suppress the activation of this cell, similarly to CD4 T cells (70, 71).

MODULATION OF MACROPHAGE PHENOTYPE

Macrophages are part of reproductive tissues together with epithelial and stromal cells both in humans and mice (72). Considering that reproductive organs, which are targets of *N. gonorrhoeae* infection, have a privileged immune status (73–75), one can speculate that macrophages could contribute to regulatory response. Findings of our laboratory have demonstrated that gonococcus affects murine RAW264 and its functionality. We showed a shift toward the production of IL-10 and TGF-β and inefficient up-regulation in molecules involved in antigen presentation such as MHC class II and CD86, and therefore a weak allogeneic T-cell stimulatory activity (45).

Similarly, differentiated macrophages from THP-1 and U937 cells challenged with *N. gonorrhoeae* showed induction of IL-10 and low levels of IL-12 (27). In this line, we have also demonstrated that MDM challenged with *N. gonorrhoeae* were differentiated toward an M2 profile, expressing the CD163 marker, inducing anti-inflammatory cytokines and inhibitory surface molecules with a low capacity to stimulate CD4 T cells proliferation (46). Regarding this, it is crucial to implement studies that characterize the phenotype of macrophages in a murine model or the genital tract of the patient.

OPPORTUNITIES AND CHALLENGES OF THERAPEUTICS TO CONTROL IMMUNE MANIPULATION

Due to multi-drug resistance problems related to gonorrhea, researchers and clinicians are dedicated to making preventive vaccines. However, the capacity to create an effective vaccine has not been possible yet due to the characteristics of the immune response against *N. gonorrhoeae* (76). Today, the development of gonorrhea vaccines has not been effective. Only one study has shown reduced rates of gonorrhea infection in patients immunized with outer membrane vesicle meningococcal B vaccine (MeNZB) with an effectiveness of 31% (77). However, these findings are the first evidence in humans lately. Thus, other types of treatments become necessary. Liu et al. assessed an intravaginal treatment using IL-12–loaded microspheres in a murine model of infection. Microencapsulated IL-12 transforms the infection in a "live vaccine" that triggers the

production of local and systemic gonococcus-specific antibodies (78). This method was also evaluated together with a non-living gonococcal vaccine based on gonococcal outer membrane vesicles (OMV) showing an elicitation of long-term humoral protective immunity driven by a Th1 response, which was effective against antigenically diverse strains of *N. gonorrhoeae* (79). Besides, the blockade of TGF-β1 and IL-10 has been useful to reverse *N. gonorrhoeae*-mediated suppression of Th1 and Th2 responses and it facilitates the development of specific protective immunity (11, 80). Moreover, Youssef et al. hypothesized the use of curcumin and vitamin D to "rescue" the immunity, by counteracting the different ways used by *N. gonorrhoeae* to evade the immune response (76). Considering that gonococcus affects macrophages polarization toward M2 phenotype (42), a putative treatment which would reverse M2 to M1 polarization is likely to be beneficial, since M1 polarization is associated with control of acute infections by many intracellular bacteria (81). In this regard, Na et al. blocked M-CSF-induced M2 macrophages differentiation using a COX-2 inhibitor Etodolac driving pro-inflammatory activities in human and murine macrophages and proposing it as a therapy for induction of enhanced anti-tumor immunity (82). In this line, we think that the M2 phenotype promoted by gonococcus could be reverted using COX-2 inhibitor. We also showed no significant differences in IL-1β levels in MDM infected cells compared to non-infected MDM cells, suggesting that *N. gonorrhoeae* could trigger insufficient IL-1β levels to activate innate immune response (83). Then, we were able to promote the IL-1β processing and release using exogenous ATP. Interestingly, production of IL-1β did not correlate with the activation of inflammasome-mediated caspase-1, suggesting that ATP could be acting at the level of mechanisms related to vesicle trafficking or pore formation.

We also showed a significant up-regulation of co-inhibitory molecule Programmed Death Ligand 1 (PD-L1) and CD86 down-regulation in macrophages upon gonococcus infection (46). Considering that in the immunological synapse the outcome of the immune response relies at least on balance between positive (CD86) and negative (PD-L1) co-stimulation signals, we hypothesize that an increase in PD-L1 expression, particularly in macrophages, represents a strategy used by *N. gonorrhoeae* to inhibit previously activated T cells at the later stages of the immune response in peripheral tissues. It could be reversed by PD-L1 blockade, as it has been shown for the treatment of severe chronic infectious diseases such as LCMV, HIV, HCV, HBV, *C. trachomatis,* and *T. crassiceps* infection (84–87).

CONCLUSION

Until now, much of the research associated with modulation of innate immune cell response has been focused on gonococcus-PMN interaction, and many advances in this field have been achieved. Nevertheless, the interaction of *N. gonorrhoeae* with the resident or recruited macrophages is less well understood. Our current knowledge of the *N. gonorrhoeae* interactions with macrophages shows the capacity of gonococcus to avoid the response to an infectious challenge (phagocytosis and effective killing) and the elicitation of acquired immune response (**Figure 1**). Although we are still investigating which are the molecules from *N. gonorrhoeae* that confer resistance to macrophage, we have advanced in many questions about modulation of macrophage response and its role in gonococcal pathogenesis. There are still questions associated with the signaling pathways and cellular mechanisms related to inflammatory response, as well as the effects in the T cell activation and differentiation. Accessibility to mouse and human macrophages and the capacity of genetic or pharmacological manipulation will facilitate the studies addressed to find potential targets to be exploited with new therapeutic approaches beyond antimicrobial drugs.

AUTHOR CONTRIBUTIONS

AE conceived and designed the mini review and wrote the mini review. PR contributed to the writing and critically revised the paper. CA-C critically revised the paper. All authors read and approved the submitted version.

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Structural Basis for Evasion of Nutritional Immunity by the Pathogenic *Neisseriae*

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The pathogenic *Neisseria* species are human-adapted pathogens that cause quite distinct diseases. *Neisseria gonorrhoeae* causes the common sexually transmitted infection gonorrhea, while *Neisseria meningitidis* causes a potentially lethal form of bacterial meningitis. During infection, both pathogens deploy a number of virulence factors in order to thrive in the host. The focus of this review is on the outer membrane transport systems that enable the *Neisseriae* to utilize host-specific nutrients, including metal-binding proteins such as transferrin and calprotectin. Because acquisition of these critical metals is essential for growth and survival, understanding the structures of receptor-ligand complexes may be an important step in developing preventative or therapeutic strategies focused on thwarting these pathogens. Much can also be learned by comparing structures with antigenic diversity among the transporter sequences, as conserved functional domains in these essential transporters could represent the pathogens' "Achilles heel." Toward this goal, we present known or modeled structures for the transport systems produced by the pathogenic *Neisseria* species, overlapped with sequence diversity derived by comparing hundreds of neisserial protein sequences. Given the concerning increase in *N. gonorrhoeae* incidence and antibiotic resistance, these outer membrane transport systems appear to be excellent targets for new therapies and preventative vaccines.

Keywords: nutritional immunity, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, transition metals, iron and zinc piracy

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INTRODUCTION

The pathogenic *Neisseriae* include two obligate human pathogens: *Neisseria gonorrhoeae* and *Neisseria meningitidis*. Although these bacteria cause very different diseases, they are so similar at the genome-sequence level that they should be referred to as the same species according to threshold values currently recommended to differentiate species (Kim et al., 2014). *N. gonorrhoeae* causes the common sexually transmitted infection (STI) gonorrhea, which is associated with low mortality but high morbidity, particularly among women. Uncomplicated infections include urethritis in men and cervicitis in women; however, asymptomatic

infections are common among women and, thus, the pathogen has the opportunity to ascend into the upper reproductive tract to cause salpingitis, pelvic inflammatory disease, ectopic pregnancy or infertility (Hook and Handsfield, 2008). *N. gonorrhoeae* can escape the genital tract entirely, resulting in disseminated gonococcal infection, for which the predominant symptoms are septic arthritis and dermatological eruptions. Due to the asymptomatic nature of many infections in women, these later manifestations are more likely to occur in females (Hook and Handsfield, 2008). Antibiotic resistance among gonococcal isolates is a serious and emergent problem. Of all the isolates collected in the United States in 2017, nearly 50% of isolates are resistant to one or more antibiotics and over 5% of isolates are resistant to at least three antimicrobial agents tested (CDC, 2017). Current therapy recommendations are to treat simultaneously with two drugs: ceftriaxone and azithromycin (CDC, 2015). However, resistance to both drugs has already emerged and therapy failures with this dual-drug approach have recently occurred (Eyre et al., 2018). Gonococcal infections are not protective and thus persons who suffer from this infection are not protected from subsequent episodes (Hedges et al., 1998, 1999). Finally, even after decades of research, there is no vaccine to prevent gonorrhea.

By contrast, *N. meningitidis* can cause rapidly fatal meningitis or meningococcemia. While colonization of the nasopharynx by *N. meningitidis* is common (referred to as carriage), dissemination to the blood or cerebrospinal fluid can be lethal if not treated quickly and appropriately with antimicrobial drugs (Thompson et al., 2006). Fortunately, antibiotic drug resistance has not emerged in *N. meningitidis,* as it has among gonococcal isolates, and penicillin-G is still the drug of choice for treatment of meningococcal infections (Nadel, 2016). In well-resourced countries, cases of meningococcal disease have been decreasing in frequency due to vaccination with efficacious vaccines. Recently, newly licensed vaccines to protect against serogroup B *N. meningitidis* strains have been recommended to high-incidence groups, leading to a further decline in meningococcal disease cases (Atkinson et al., 2016).

Pathogenic *Neisseria* species utilize similar virulence factors to cause disease in their human hosts. A major difference between *N. gonorrhoeae* and *N. meningitidis* is that the former lacks a polysaccharide capsule, which enables the latter to more effectively evade phagocytosis and desiccation (reviewed in Lewis and Ram, 2014). Both *N. gonorrhoeae* and *N. meningitidis* produce pili that are required for adherence to mucosal epithelia, and a series of phase-variable adhesins and invasins, called Opacity or Opa proteins (reviewed in Merz and So, 2000). Other key virulence factors include various other adhesins, an IgA protease, toxic lipooligosaccharide, and a series of outer membrane proteins involved in serum resistance (for review see Quillin and Seifert, 2018). Another key virulence trait is the ability to acquire the necessary nutrients for bacterial replication in the face of the host's attempts to starve the pathogen of these building blocks. This innate immunity defense is often referred to as nutritional immunity (Kochan, 1973; Weinberg, 1977, 1978), which is the subject of this review.

NUTRITIONAL IMMUNITY AND METALS

N. gonorrhoeae and *N. meningitidis* are both obligate human pathogens, which necessitates the requirement for these invaders to obtain all necessary nutrients for growth and pathogenesis from within their human hosts. Humans devote a considerable armament to sequestering many of these necessary nutrients for bacterial growth, particularly metals, into niches that pathogens cannot access. This process, which effectively inhibits the growth of human pathogens due to nutrient deprivation, has been referred to as "nutritional immunity" (Kochan, 1973; Weinberg, 1977, 1978). The best characterized forms of nutritional immunity relate to iron sequestration by the host, including the production of proteins such as lactoferrin (Weinberg, 2001) and lipocalin (Miethke and Skerra, 2010), which actively absorb iron and ferric siderophores, respectively. Other human proteins that maintain free iron at scarce levels in human hosts include transferrin and hemoglobin. In the face of these metal chelating proteins, many pathogens are unable to compete for the necessary nutrient, iron, and thus bacterial growth is restricted. In a similar fashion, a recently recognized phenomenon occurs in humans attempting to sequester another metal, zinc (Liuzzi et al., 2005). Nutritional immunity proteins including calprotectin and other so-called S100 proteins actively, and with high affinity, bind to and "hide" zinc from microbial invaders. The S100 family of proteins are EF-hand proteins involved in calcium sensing and chelation and also bind other divalent cations, including zinc and manganese, with high affinity (Zackular et al., 2015). In a number of microbial systems, these proteins have been shown to be powerful suppressants of growth and therefore pathogenesis.

IRON PIRACY AND BACTERIA

In order to be successful pathogens, bacteria must overcome nutritional immunity imposed by human hosts. One of the most common approaches to acquiring sufficient iron is to deploy bacterial siderophores, which are molecular cages capable of chelating ferric iron with high affinity. The affinity of these compounds for iron is so high that it out-competes that of the human proteins produced for iron sequestration. For example, the affinity of enterobactin for ferric iron has been estimated to be 10^{-52} M (Neilands, 1981), whereas the affinity of transferrin for iron is considerably lower at 10^{-30} M (Davis et al., 1962). However, enterobactin is not an effective virulence factor in many niches due to the production of another host innate immunity protein called lipocalin. Lipocalin sequesters enterobactin so that bacterial producers do not reap the benefits of deploying this siderophore for iron uptake (Borregaard and Cowland, 2006). Some crafty pathogens, including *Salmonella* species, have evolved a parallel pathway to enterobactin biosynthesis in which they further decorate enterobactin with glucose molecules, yielding salmochelin, which effectively evades lipocalin sequestration (Feldmann et al., 2007; Muller et al., 2009). Thus, both pathogens and the human host are evolving in ways that either enable iron uptake (pathogen)

or further sequester iron (host) in the ongoing struggle for this necessary nutrient.

THE STRUCTURAL BASIS FOR IRON PIRACY BY THE *Neisseriae*

The pathogenic *Neisseriae* are somewhat unusual in that they do not produce any siderophores for metal acquisition. Instead, these stealthy pathogens rely primarily on metal extraction directly from human innate immunity proteins, including transferrin (Tf) and lactoferrin (Lf). The focus of the remainder of this review is on the structural basis by which these transporters enable the pathogenic *Neisseria* species to overcome nutritional immunity imposed by the human host.

Tf-IRON ACQUISITION SYSTEM

Transferrin-binding protein A, or TbpA (Noinaj et al., 2012), is a typical TonB-dependent transporter (TdT), which, like all others, possesses 22 transmembrane beta-strands, separated by 11 surface-exposed loops and 11 short periplasmic turns (**Figure 1**). Also, like other TdTs, TbpA has an amino-terminal plug domain of ∼150 amino acids, which effectively occludes the beta-barrel domain (Noinaj et al., 2012). At least three motifs have been identified in TbpA that are critical to the iron-uptake function of this transporter. As is true for all TdTs, an amino-terminal sequence motif known as the TonB-box is essential for interaction with the charging protein, TonB (Kenney and Cornelissen, 2002). We demonstrated that mutagenesis of the TonB-box abrogated transport function while preserving transferrin-binding capabilities (Kenney and Cornelissen, 2002). On the surface of TbpA is an extruding helix finger protruding from extracellular loop 3 (**Figure 1A**). The co-crystal structure between TbpA and human transferrin strikingly demonstrated that this helix finger projects into the cleft within the C-lobe of transferrin, which is the only lobe with which TbpA interacts to extract iron (Noinaj et al., 2012) (**Figure 1B**). This led to the hypothesis that the loop 3 helix is a critical motif with which TbpA manages to remove iron from its ligand. Cash et al. (2015) tested this hypothesis by site-directed mutagenesis and found that changing the charges of the helix or changing charged residues to alanine residues did not fully abrogate either transferrin binding or iron uptake. However, complete deletion of this motif on loop 3 did disable the transporter and prevent *N. gonorrhoeae* from growing on human transferrin as a sole source of iron (Cash et al., 2015). The third motif important for transferrin-iron internalization is within the plug domain. Noto and Cornelissen (2008) and subsequently Banerjee et al. (2012) found that the EIEYE motif in the plug (**Figures 1A–C**) was necessary for iron utilization from transferrin and moreover for direct interaction between the positively charged ion and the plug. These observations support the hypothesis that after iron is extracted from transferrin, the cation is transiently chelated by the plug. Thus, this protein is effectively a transferrin receptor and iron transporter; it is currently unclear whether TbpA is capable of transporting iron independent of its protein carrier. As shown in **Figure 1B**, this sequence motif is located in the proposed iron pathway through the TbpA barrel. After the plug is energized by TonB, and presumably moved or removed from the barrel, this change in conformation is sufficient to enable iron removal from the plug and the subsequent hand off of the cation to FbpA (not shown), which is located in the periplasm (Noinaj et al., 2012).

TbpA is very well conserved (Cornelissen et al., 2000) and not subject to high-frequency phase or antigenic variation. Gonococcal TbpA sequences share greater than 95% sequence identity and thus the choice of any single TbpA type as a vaccine antigen is expected to elicit broadly cross-reactive responses. These observations, along with the fact that TbpA is a necessary virulence factor for human infection (Cornelissen et al., 1998), lead to the conclusion that this antigen is a promising vaccine component. As shown in **Figure 1D**, sequence diversity (shown in blue) is primarily restricted to the long extracellular loops. The plug and barrel domains are very well conserved. Interestingly, even those regions that directly interact with ligand, such as the loop 3 helix, demonstrate some sequence variation. These data suggest that while the charge, conformation, and perhaps the size of the helix finger may be critical for iron extraction, the precise sequence is not. These observations are consistent with those of Cash et al. (2015) who concluded that the helix finger sequence is quite tolerant to mutation and therefore sequence variation.

Transferrin-binding protein B, or TbpB, is a lipid-modified protein (Anderson et al., 1994) that extends outward from the outer membrane via an extended anchor peptide (**Figure 2A**). Like TbpA, TbpB interacts with human transferrin via the C-lobe of the ligand (Noinaj et al., 2012). TbpB exhibits a bi-lobed structure as well (Moraes et al., 2009; Noinaj et al., 2012), with the N-lobe of TbpB forming a binding interface with the C-lobe of transferrin (**Figure 2B**). It is unclear what the function of the C-lobe of TbpB is, but this domain may, in part, be important to accomplish export of the lipidated protein to the outer leaflet of the outer membrane via the SLAM system (Hooda et al., 2017). TbpB recognizes only the ferrated form of human transferrin (Cornelissen and Sparling, 1996), enabling this protein to distinguish between the ligand that is useful for iron transport (ferrated) and that which is not (apo form). DeRocco et al. (2008) demonstrated that the presence of TbpB on the gonococcal cell surface enhanced both association with and disassociation from the TbpA transporter. The presence of TbpB enhances iron uptake from transferrin by 50% (Anderson et al., 1994), which is consistent with its observed metal selectivity and ability to form a chamber to trap extracted iron when it complexes with both TbpA and transferrin (Noinaj et al., 2012).

TbpB is considerably more antigenically variable than is TbpA (Cornelissen et al., 1997), although neither protein is subject to high-frequency variation mechanisms as are the pilin or opacity proteins. As shown in **Figure 2C**, loops of TbpB again show the greatest variation from strain to strain. However, given that the entire protein, including the beta-barrels, is surface-exposed, it is not surprising that immune pressure drives increased antigenic diversity.

Both TbpA and TbpB have been evaluated as possible vaccine targets in a series of studies by Price et al. (2004).

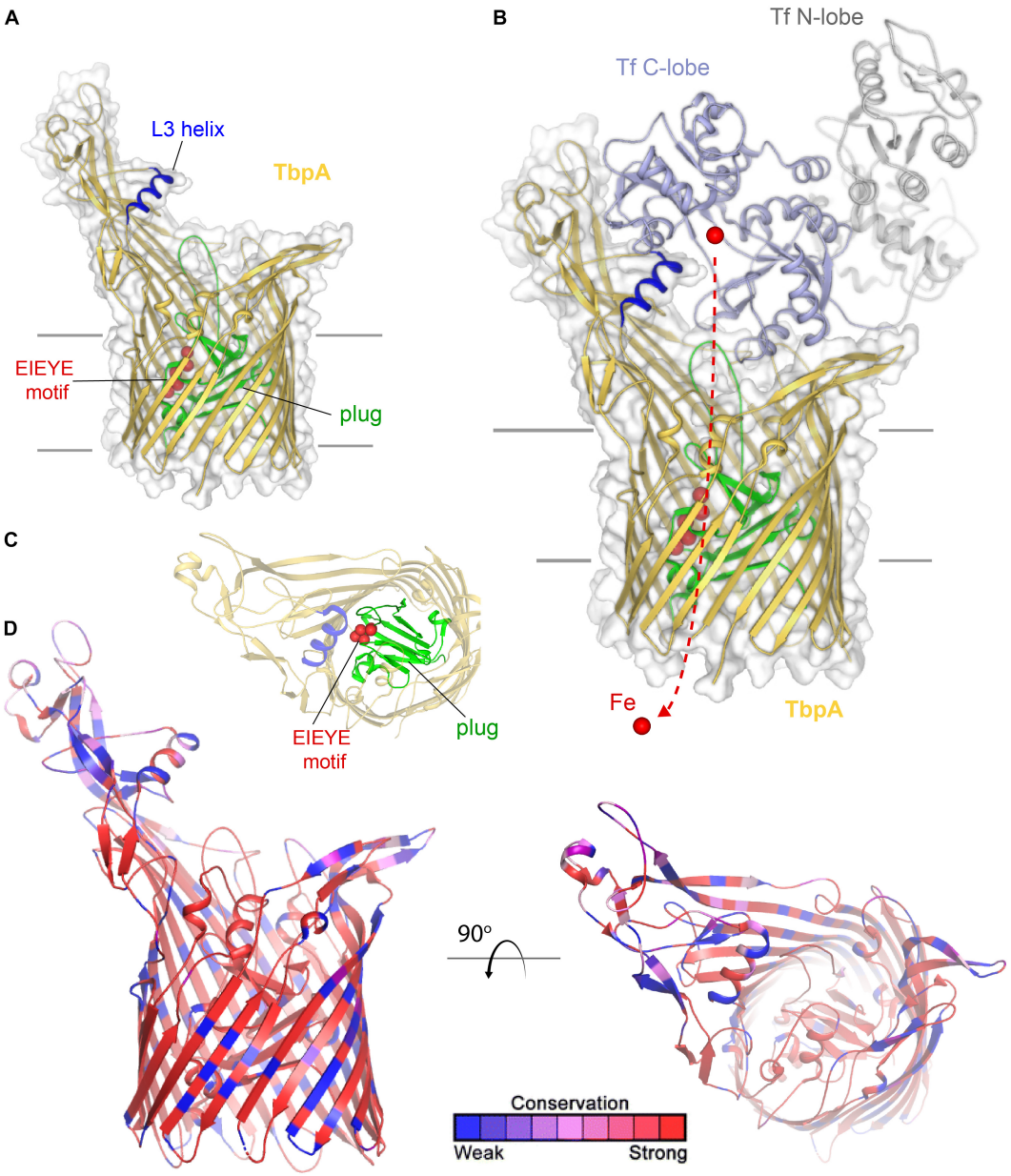

FIGURE 1 | Overall structure of TbpA and interactions with transferrin. **(A)** The structure of TbpA from *N. meningitidis* (K454) (from PDB ID 3V8X) is shown in gold cartoon. The L3 helix is shown in blue, the EIEYE motif in red, and the plug domain in green. The surface is shown in gray transparency. **(B)** The complex of TbpA with apo transferrin (PDB ID 3V8X) is shown with the C-lobe of transferrin in light blue cartoon and the N-lobe in gray cartoon. Iron (red sphere) has been modeled with the red dashed line indicating the putative route through the barrel domain of TbpA during import into the periplasm. **(C)** View looking down the barrel of TbpA showing the plug domain (green) and residues of the EIEYE motif (red spheres). **(D)** The structure of TbpA in cartoon mapping the Consurf conservation scores for *N. meningitidis* and *N. gonorrhoeae* where red is most conserved and blue is the least conserved (also see **Supplementary Figure 1**).

Natural infections generate little to no anti-Tbp immune responses, and considering that infections are not protective, these observations engender optimism that generating an anti-Tbp immune response by vaccination could lead to protection. Immunization of mice with full-length Tbp proteins conjugated to the B-subunit of cholera toxin generated immune responses with potentially protective activities (Price et al., 2007). Both mucosal and serum IgA and IgG antibodies were generated in this study. Serum antibodies were capable of fixing human complement and killing Tbp-producing gonococci. Interestingly, vaccination of mice with a cocktail of both TbpA and TbpB conjugates resulted in complement-mediated killing of both the homologous strain and heterologous strains of *N. gonorrhoeae* (Price et al., 2007). Finally, in a third study, Price et al. demonstrated that genetic chimeras consisting of the amino-terminal half of TbpB plus loop 2 of TbpA fused to the A2

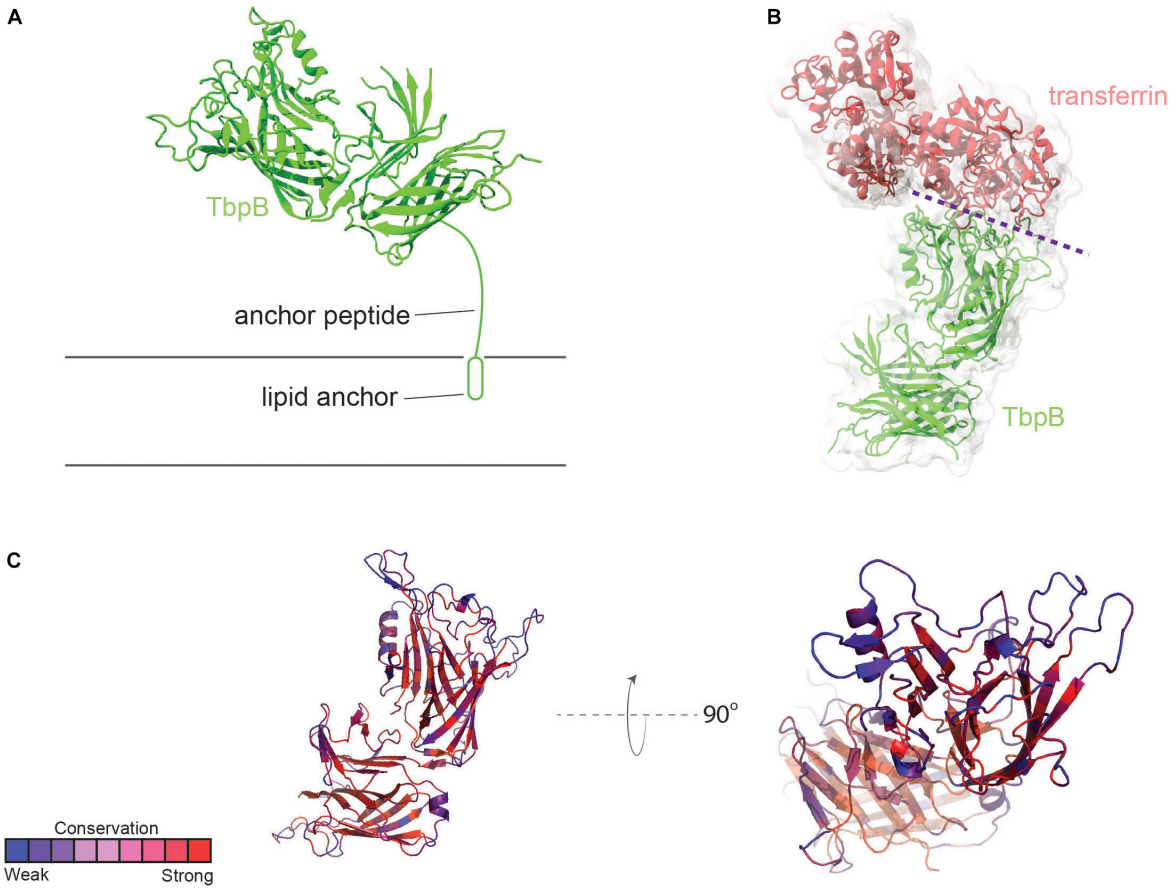

FIGURE 2 | Overall structure of TbpB and interactions with transferrin. **(A)** *N. meningitidis* M982 TbpB (from PDB ID 3VE2) is shown in green cartoon; anchored to the membrane by a lipid moiety. **(B)** The complex of TbpB with transferrin (PDB ID 3VE2) is shown (binding interface represented with dashed purple line). **(C)** Conservation scores of specific residues from AL2CO are typically high within the core barrel and handle (red). Loop regions involved in binding to human transferrin are typically subject to a more intense selection pressure (blue) (also see **Supplementary Figure 2**).

subunit of cholera toxin generated both mucosal and serum immune responses. Similarly, the serum antibodies were cross-bactericidal and additionally the pooled mucosal secretions from vaccinated mice were capable of blocking the use of human transferrin by *N. gonorrhoeae in vitro* (Price et al., 2005). These studies cumulatively indicate that the more variable TbpB elicits a vigorous but sequence-specific immune response while addition of TbpA to the vaccine formulation results in cross-reactive, potentially protective immune responses.

Lf-IRON ACQUISITION SYSTEM

Much less is known about lactoferrin binding protein A, or LbpA, including its precise crystal structure. However, given the sequence conservation between TbpA and LbpA, high confidence can be afforded to models that demonstrate important structural features of LbpA (**Figure 3**). The motifs that have been demonstrated to be important for TbpA function can also be identified in LbpA. These motifs include the TonB-box (not shown), the L3 helix and the EIEYE motif. In fact, the EIEYE motif was initially identified by scanning the plug domain for the following residues: conserved in all TbpAs, conserved in all LbpAs, and capable of coordinating iron based on negative charges (Noto and Cornelissen, 2008). Although not directly tested, these motifs are anticipated to function analogously in LbpA to their demonstrated functions in TbpA. Like TbpA, LbpA is quite well conserved (Adamiak et al., 2015). Despite its conservation among strains, LbpA and its lipoprotein partner, LbpB (see next section), are not thought to be particularly good vaccine candidates. In some studies, only approximately half of the gonococcal strains express LbpA (Anderson et al., 2003) and, in many additional isolates, LbpB is phase off due to slipped-strand mispairing (Anderson et al., 2003). Despite these observations, there is concern that if the Tbps are targeted for vaccine development, acquired immunity could drive selection for more strains able to produce the Lbps and therefore utilize lactoferrin as a sole iron source. The potential redundancy in these iron transport systems has been experimentally verified in human infection studies. In studies conducted by Anderson et al. (2003), a genetically engineered strain that could produce the Lbp proteins, but could not produce the Tbps, was capable

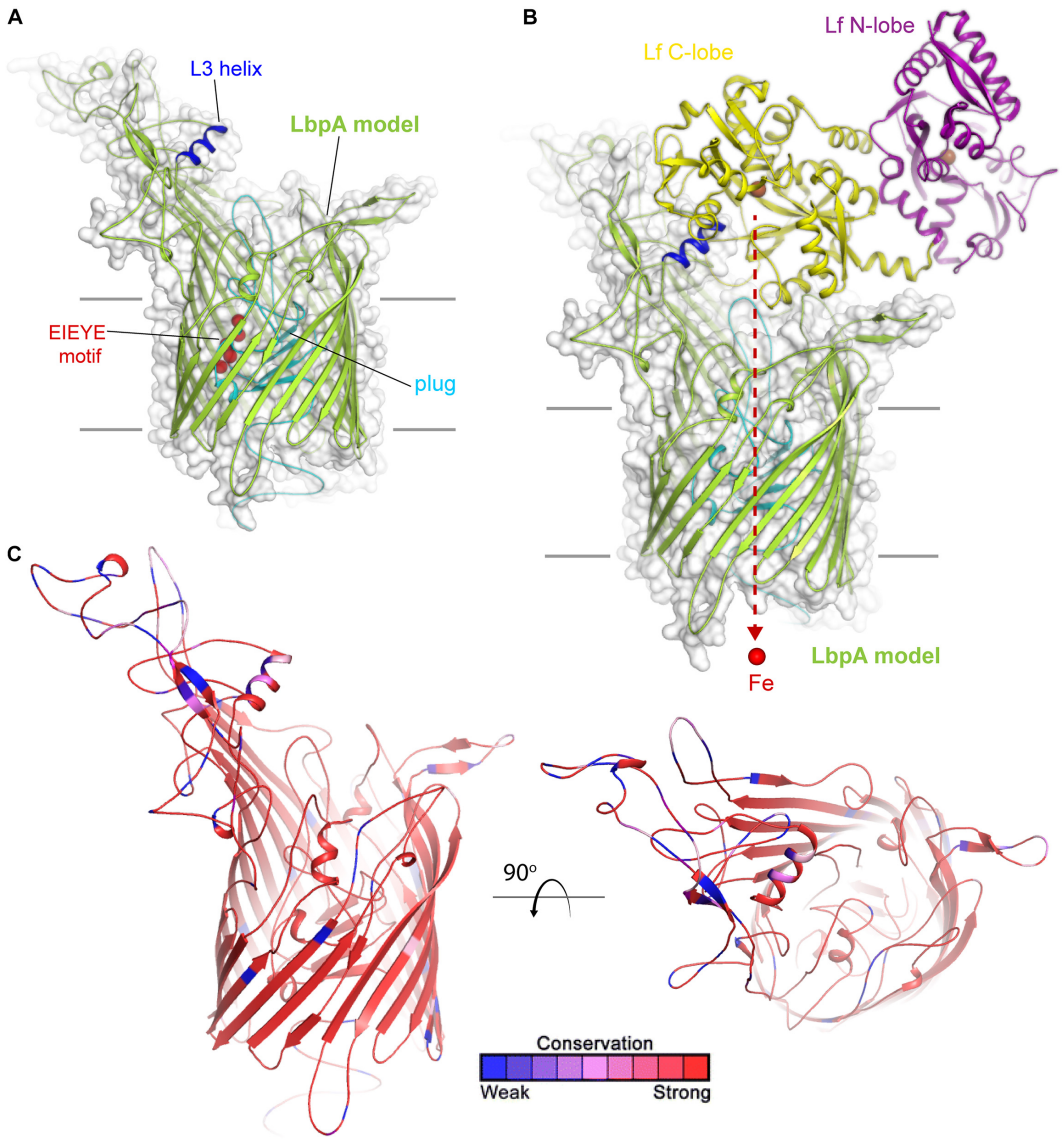

FIGURE 3 | Insights into the structure of LbpA and interactions with lactoferrin. **(A)** Model (SWISS-MODEL) for LbpA from *N. meningitidis* (MC58) is shown in green cartoon. The L3 helix is shown in blue, the EIEYE motif in red, and the plug domain in cyan. The surface is shown in gray transparency. **(B)** Model for the complex of LbpA with holo lactoferrin (PDB ID 1LFG) is shown with the C-lobe of lactoferrin in yellow cartoon and the N-lobe in purple cartoon. This model is based on the reported TbpA-apo transferrin complex structure. Iron (red sphere) has been modeled with the red dashed line indicating the putative route through the barrel domain of LbpA during import into the periplasm. **(C)** The structure of LbpA in cartoon mapping the Consurf conservation scores for *N. meningitidis* and *N. gonorrhoeae* where red is most conserved and blue is the least conserved (also see **Supplementary Figure 3**).

of causing experimental infection. Moreover, a strain producing both iron acquisition systems was more competitive than a strain producing only one of these systems (Anderson et al., 2003). These studies suggest that perhaps the best approach will be to target multiple iron transport systems in a cocktail-type vaccine.

Lactoferrin-binding protein B, or LbpB (**Figure 4**) is, like its transferrin-binding homolog, a lipoprotein that is entirely exposed on the cell surface and tethered to the cell by a lipid anchor (Noinaj et al., 2013). Similar to LbpA, no full-length structure has yet been reported for LbpB; however, structures of the N-lobe only have been determined (Brooks et al., 2014). As expected based upon homology (Noinaj et al., 2013), the conformation of this protein resembles that of TbpB, including a bilobed structure with both barrel and handle domains (**Figures 4A,B**). Similar to TbpB, it is anticipated that the N-lobe of LbpB interacts exclusively with the C-lobe of human lactoferrin and thus increases the efficiency of iron transport from lactoferrin. Sequence diversity for LbpB resembles that of TbpB in that the solvent-exposed loops are highly diverse (**Figure 4C**). However, the beta-barrels are better conserved than those in TbpB, perhaps reflecting the fact that this protein is often not produced *in vivo*, thus an immune response does not drive

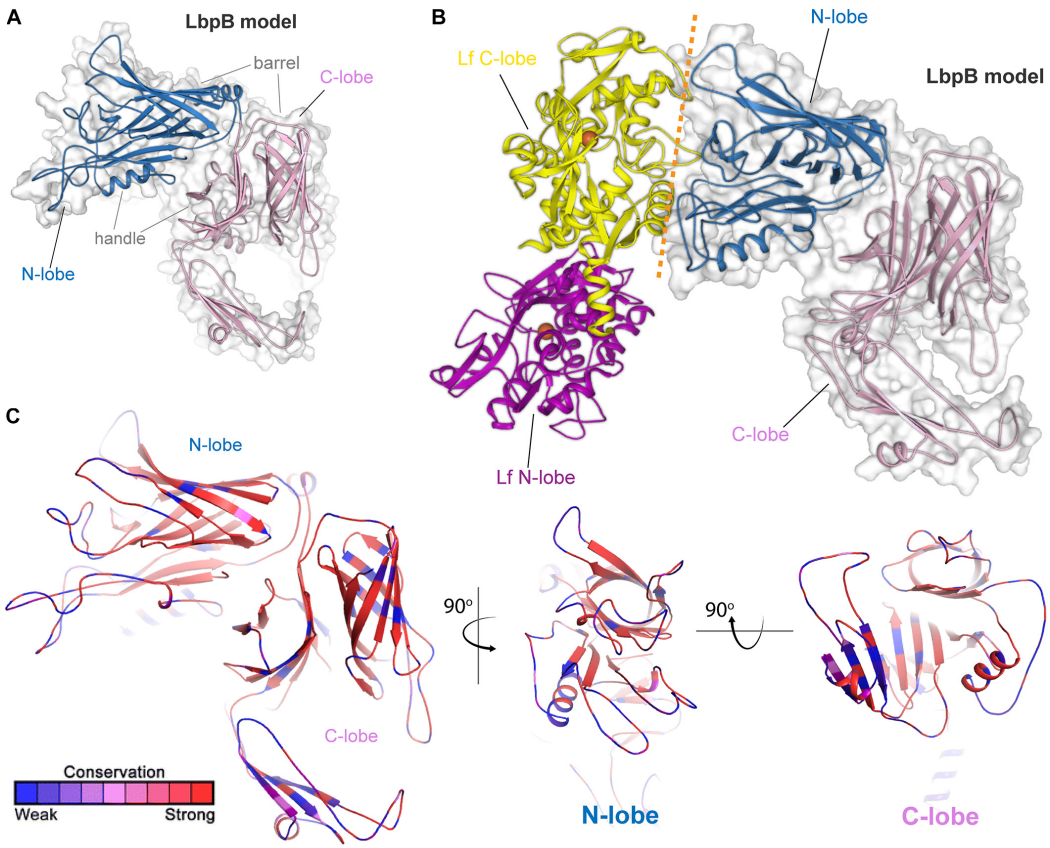

FIGURE 4 | Insights into the structure of LbpB and interactions with lactoferrin. **(A)** Model (SWISS-MODEL and PDB ID 4U9C) for LbpB (MC58) from *N. meningitidis* is shown in blue (N-lobe) and pink (C-lobe) cartoon. The barrel and handle domains are conserved motifs and indicated for each lobe. The surface is shown in gray transparency. **(B)** Model for the complex of LbpB with lactoferrin (PDB ID 1LFG) is shown with the C-lobe of lactoferrin in yellow cartoon and the N-lobe in purple cartoon. Lactoferrin-bound irons are indicated as orange spheres with the red dashed line indicating putative binding interface between lactoferrin and LbpB. This model is based on the reported TbpB-holo transferrin complex structure (PDB ID 3VE1). **(C)** Model of LbpB in cartoon mapping the Consurf conservation scores for *N. meningitidis* and *N. gonorrhoeae* where red is most conserved and blue is the least conserved (also see **Supplementary Figure 4**). Orthogonal views of the N-lobe and C-lobe are shown for comparison.

antigenic diversity. Lastly, *N. meningitidis* selectively expresses NalP, which is a phase-variable autotransporter/lipoprotein hybrid that houses a subtilisin-like protease domain responsible for releasing LbpB from the bacterial membrane (Roussel-Jazede et al., 2010; Del Tordello et al., 2014). NalP is thus believed to help divert host immune factors away from the bacterial cell surface during invasive infection, again suggesting that LbpB is likely a poor vaccine candidate for protection against *N. meningitidis*. NalP is, however, exclusive to *N. meningitidis* and thus whether similar evasion mechanisms exist in *N. gonorrhoeae* is currently unknown.

Hb-IRON ACQUISITION SYSTEMS

The genomes of the pathogenic *Neisseria* contain the genetic capacity to express two distinct hemoglobin acquisition systems. HmbR or HmbR plus HpuAB are most commonly produced by *N. meningitidis* strains (Harrison et al., 2013); however, all *N. gonorrhoeae* strains characterized to date only produce HpuAB since the *hmbR* gene is a pseudogene (Harrison et al., 2013). Moreover, the *hpuAB* genes in both pathogenic species are subject to phase variation (Chen et al., 1998; Lewis et al., 1999). Interestingly, several species of commensal *Neisseriae* do not have the repeat region upstream of *hpuAB*, resulting in expression that is not subject to high-frequency phase variation (Harrison et al., 2013).

The *hpuAB* locus encodes two proteins, HpuA and HpuB, which are capable of binding to hemoglobin (**Figure 5**) and also to the hemoglobin:haptoglobin complex (Lewis et al., 1997) (not shown). HpuA is the lipoprotein component of the system (Lewis et al., 1997) and is anticipated to take on a conformation that is fully extended outward from the cell surface. HpuB is the TdT component that enables the entry of heme after its extraction from hemoglobin. Unlike the transferrin and lactoferrin transport systems, ligand binding and utilization requires the participation of both HpuA and HpuB (Chen et al., 2002). The HpuA protein is roughly half the size of the other lipoprotein transport components but structurally resembles the C-lobe of both TbpB and LbpB (Wong et al., 2015). Much less is

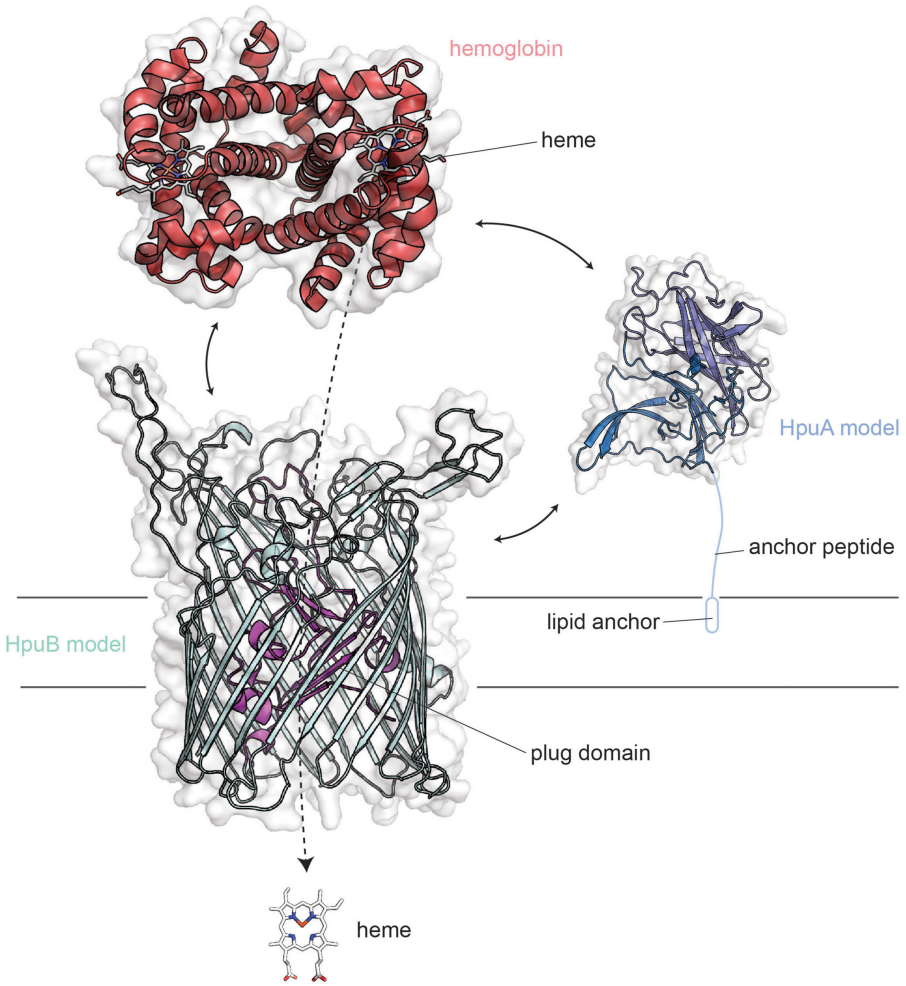

FIGURE 5 | Overall structures of HpuA/B visualized with human hemoglobin. HpuA model (blue; SWISS-MODEL) is shown anchored to the membrane via a lipidated anchor peptide. HpuB (teal; SWISS-MODEL) is shown embedded within the outer membrane. These two proteins act in concert to form a ternary complex and pirate heme from host hemoglobin (red).

known about the structure-function relationships of the HpuAB proteins; however, the crystal structure has been reported for a related HpuA from *Kingella* (Wong et al., 2015), which was used to form the homology model for neisserial HpuA, shown in **Figure 5**.

HpuA and B are produced by both pathogenic *Neisseria* species but are also subject to high-frequency phase variation (Lewis et al., 1997, 1999). Some virulent clonal populations of *N. meningitidis* possess both HpuAB and HmbR gene loci suggesting there may be a benefit to producing both systems, particularly in systemic infections (Harrison et al., 2013). However, no *N. gonorrhoeae* strains produce HmbR (Harrison et al., 2013), and in all of the infected men tested, the variants isolated *in vivo* were phase-off for the *hpuAB* system (Anderson et al., 2001). In the same study, Anderson et al. found that most women with a localized infection by *N. gonorrhoeae* similarly were culture positive for isolates that were phase-off for the *hpuAB* system. The only group from which phase-on *hpuAB* variants were isolated were women infected during the first 2-weeks of their menstrual cycle (Anderson et al., 2001). These observations cumulatively suggest that during localized *N. gonorrhoeae* infections, the HpuAB hemoglobin acquisition system is not critical for survival or pathogenesis. However, when hemoglobin is plentiful, such as during menses, this system is selected for as the ligand is available and could potentially perpetuate the infection (Anderson et al., 2001). In fact, ascending infections in women are associated with the onset of menses (Jossens et al., 1996; Korn et al., 1998; Holder, 2008).

HmbR (**Figure 6**) is only produced by some *N. meningitidis* isolates and a few commensal *Neisseria* species (Harrison et al., 2013). Like the HpuAB system, HmbR is subject to high-frequency phase variation by slipped-strand mispairing (Lewis et al., 1999). This protein is a TdT, which unlike those discussed above, does not have a lipoprotein partner. Possible contributions for the lipoprotein components of these systems are: an immunogenic shield for the conserved transporter

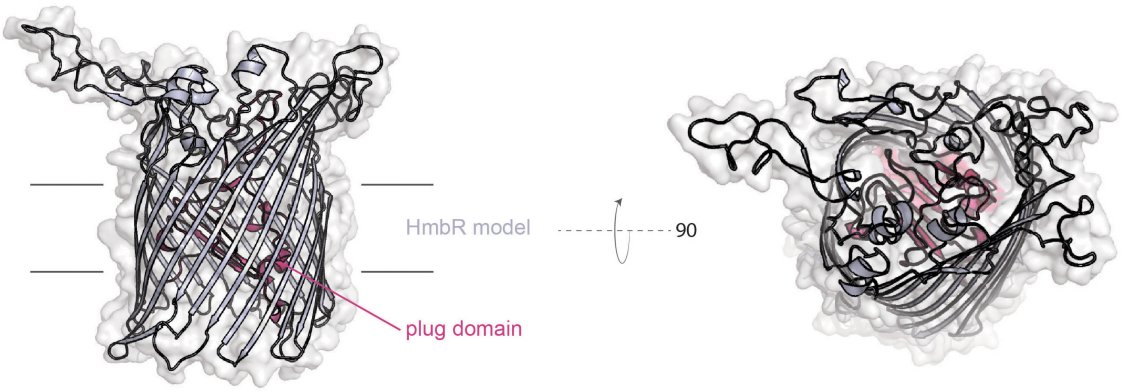

FIGURE 6 | Model of *N. meningitidis* (z4242) HmbR created with SWISS-MODEL.

(Price et al., 2005), and to impose specificity for the ligand that is useful for metal uptake (Cornelissen and Sparling, 1996; Noinaj et al., 2012). The fact that HmbR can function alone without a lipoprotein partner suggests that *in vivo* selection of hemoglobin for heme uptake does not require discrimination between the loaded form and the apo form. Alternatively, the absence of a lipoprotein component could suggest that immunological shielding is not as necessary to hide this protein from immune surveillance. A meningococcal mutant unable to produce HmbR was defective in an infant rat model of infection (Stojiljkovic et al., 1995); however, a similar mutant was not attenuated for growth in human blood, although a *tbpAB* mutant was not capable of growth in human blood (Bidmos et al., 2015). These data suggest that the use of transferrin in human blood is preferred over hemoglobin by the meningococcus, perhaps because hemoglobin is less available under normal conditions without significant red blood cell lysis.

SIDEROPHORE-IRON ACQUISITION SYSTEM

FetA (formerly called FrpB) is a TdT (**Figure 7**) than enables internalization of siderophores that the *Neisseria* species themselves do not have the capacity to produce. Originally named for its ability to transport ferric enterobactin (Carson et al., 1999), Hollander et al. demonstrated that this transporter has a broader ligand specificity than first realized (Hollander et al., 2011). FetA can internalize not only ferric-enterobactin, but also ferric-salmochelin, and dihydroxybenzoate monomers of these siderophores (Hollander et al., 2011). Recently, the crystal structure of FetA (Saleem et al., 2013) demonstrated two interesting features. First, consistent with the broad ligand specificity, free iron was directly coordinated to FetA in the crystal structure (Saleem et al., 2013) (**Figure 7A**). Second, as with the *Escherichia coli* enterobactin transporter, FetA is suggested to form a trimer (Saleem et al., 2013) (**Figure 7B**). The authors concluded that FetA is an iron transporter and has the potential to take up unchelated iron in the absence of siderophore (Saleem et al., 2013). TbpA similarly has the capacity to interact with the iron cation and could also contribute to free iron internalization. However, *in vivo* free iron concentrations are exceedingly low, thus it seems unlikely that iron internalization without extraction from some chelating or binding entity is physiologically relevant.

Saleem et al. (2013) demonstrated that conserved residues responsible for coordination of free iron to FetA are also found in similar positions in heme transporters and in other FetA homologs in all of the pathogenic *Neisseria*. However, a hypervariable loop and helix (**Figure 7C**) is located near this iron binding pocket. The authors of this study (Saleem et al., 2013) suggest that the helix essentially shields the vulnerable iron binding residues from immune detection, and further, that the structure of the helix is maintained despite antigenic diversity which is evoked by immunological pressure.

Similar to the hemoglobin and lactoferrin transporters, expression of the *fetA* gene is subject to high-frequency phase variation (Carson et al., 2000). But unlike in the previous cases, the slipped-strand in the *fetA* gene lies upstream within the promoter region. An increase or decrease in the number of C residues in the promoter, changes the distance between the −10 and −35 elements and therefore changes the strength of the promoter (Carson et al., 2000). Thus, the variation deployed in this system is more like a rheostat than a light switch.

Zn-ACQUISITION SYSTEMS

TdfH (CbpA) (**Figure 8**) is expressed by both of the pathogenic *Neisseria* species but by very few commensal *Neisseria* species. TdfH is a TdT that shows both sequence similarity to TbpA and HasR from *Serratia marcescens* (**Figure 8A**). The homolog of this protein was named CbpA by Stork et al. (2013) when they demonstrated that this transporter bound to the human protein, calprotectin (**Figure 8B**). Calprotectin is found in extremely high concentrations in neutrophils (Zackular et al., 2015) and also in neutrophil NETs (Jean et al., 2016). This innate immunity protein sequesters metals, including zinc and manganese, and in doing so inhibits microbial growth (Zackular et al., 2015). The ability to overcome this sequestration by the pathogenic *Neisseria* species enables them to usurp nutritional immunity and in fact take

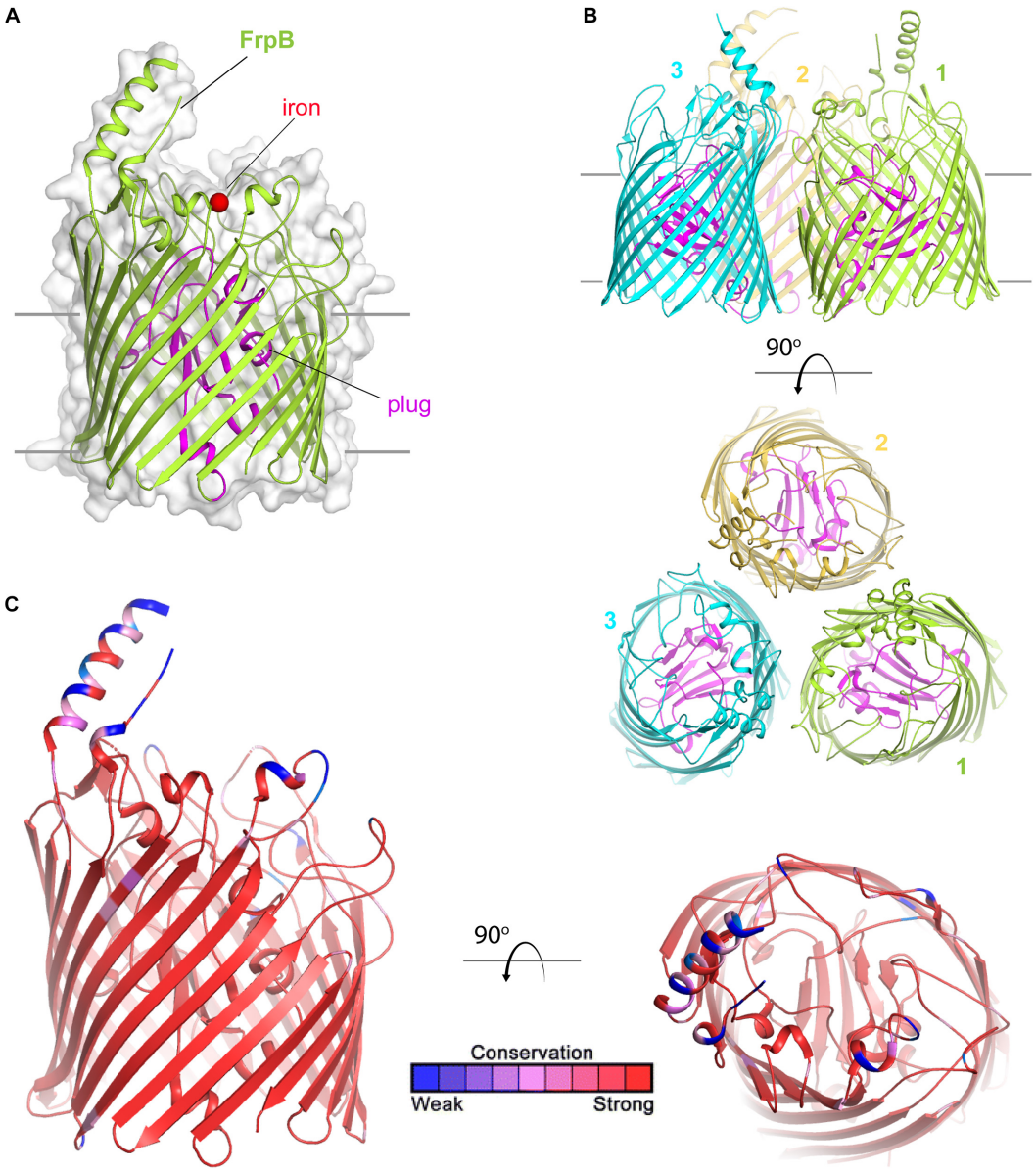

FIGURE 7 | Overall structure of FetA (FrpB). **(A)** The structure of FetA from *N. meningitidis* (Variant F5-1) (from PDB ID 4AIQ) is shown in green cartoon. The bound iron is shown as a red sphere and the plug domain in magenta. The surface is shown in gray transparency. **(B)** The trimeric form of FetA (Variant F3-3) (PDB ID 4AIP) is shown in cartoon with each monomer in a different color. The plug domains are in magenta. **(C)** The structure of FetA in cartoon mapping the Consurf conservation scores for *N. meningitidis* and *N. gonorrhoeae* where red is most conserved and blue is the least conserved (also see **Supplementary Figure 5** for neisserial FetA).

advantage of this prevalent protein as a metal source. In fact, Jean et al. (2016) demonstrated that gonococcal TdfH enabled calprotectin binding and subsequent zinc incorporation into the cell in a TdfH-dependent manner. In addition, the same study demonstrated that a mutant unable to produce TdfH was less able to survive within neutrophil NETs, suggesting that TdfH is an important virulence factor. Unlike many of the other transport systems deployed by the pathogenic *Neisseria*, TdfH does not have a lipoprotein component partner. Furthermore, TdfH is not subject to variation via slipped-strand mispairing or any other high frequency mechanism. There is some variation from strain to strain among the *Neisseriae*, but the amino acid changes are modest and primarily limited to hypothetically surface-exposed loops (**Figure 8C**).

TdfJ (ZnuD) (**Figure 9**) is a highly conserved TdT that is also expressed by both of the pathogenic *Neisseria* species, but unlike TdfH, TdfJ is also found ubiquitously amongst the commensal *Neisseriae*. Stork et al. (2010) first characterized the meningococcal homolog of this protein as contributing to zinc uptake, hence its appellation ZnuD, and this function was recapitulated for TdfJ by Jean et al. (2016), by showing that TdfJ contributes to gonococcal growth in Zn-restricted

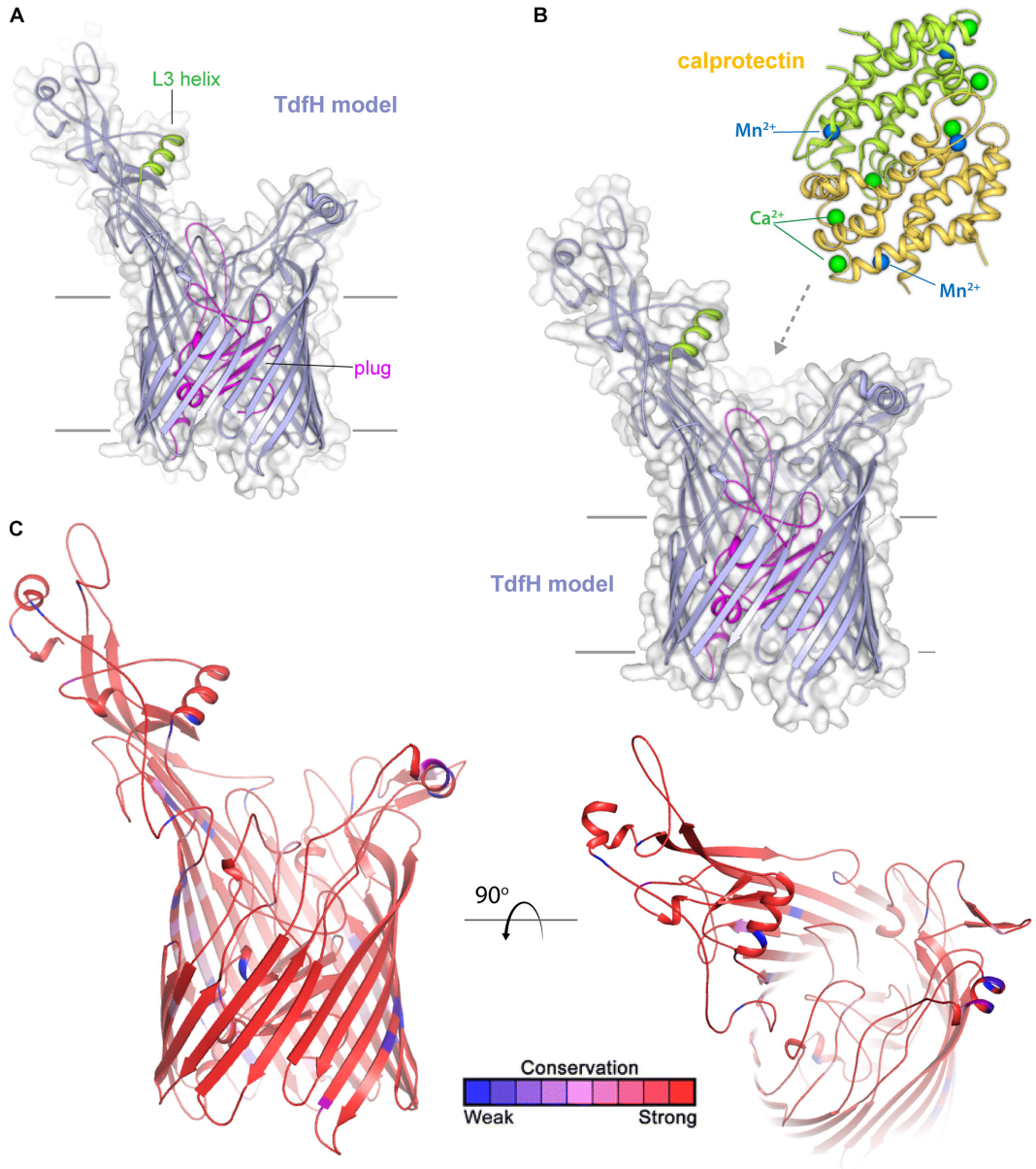

FIGURE 8 | Insights into the structure of TdfH and interactions with calprotectin. **(A)** Model (Phyre; based on TbpA) for TdfH from *N. gonorrhoeae* (FA1090) is shown in violet cartoon. The L3 helix is shown in green and the plug domain in magenta. The surface is shown in gray transparency. **(B)** Model of TdfH in proximity with calprotectin (PDB ID 4GGF) shown in green/yellow cartoon. Calcium ions are shown as green spheres and manganese metals are shown in blue spheres; zinc has been shown to replace the manganese metals with no overall structural changes to calprotectin (personal communication). While the molecular interactions between TdfH and calprotectin have not been reported, the interaction shown here is loosely modeled based on work with other transporters (PDB IDs 3CSN and 3V8X). **(C)** The model of TdfH in cartoon mapping the Consurf conservation scores for *N. meningitidis* and *N. gonorrhoeae* where red is most conserved and blue is the least conserved (also see **Supplementary Figure 6**).

conditions. Further investigation of TdfJ revealed that this protein is able to bind to human S100A7, from which it facilitates zinc removal and subsequent internalization by the gonococcus (Maurakis et al., 2019). S100A7, like the TdfH ligand calprotectin, inhibits growth of many microbes by means of metal sequestration (reviewed by Zackular et al., 2015). S100A7 is highly enriched in human epithelial tissue, including those of the oral and genital mucosa, and indeed a meningococcal mutant unable to produce the TdfJ homolog, ZnuD, was defective for survival within epithelial cells (Kumar et al., 2012). While it is not currently known whether ZnuD shares the S100A7 binding ability of TdfJ, these results suggest that interaction with S100A7 may play a critical role in establishing infection.

Similar to TdfH, TdfJ lacks a lipid-modified accessory protein like TbpB; however, the crystal structure of ZnuD

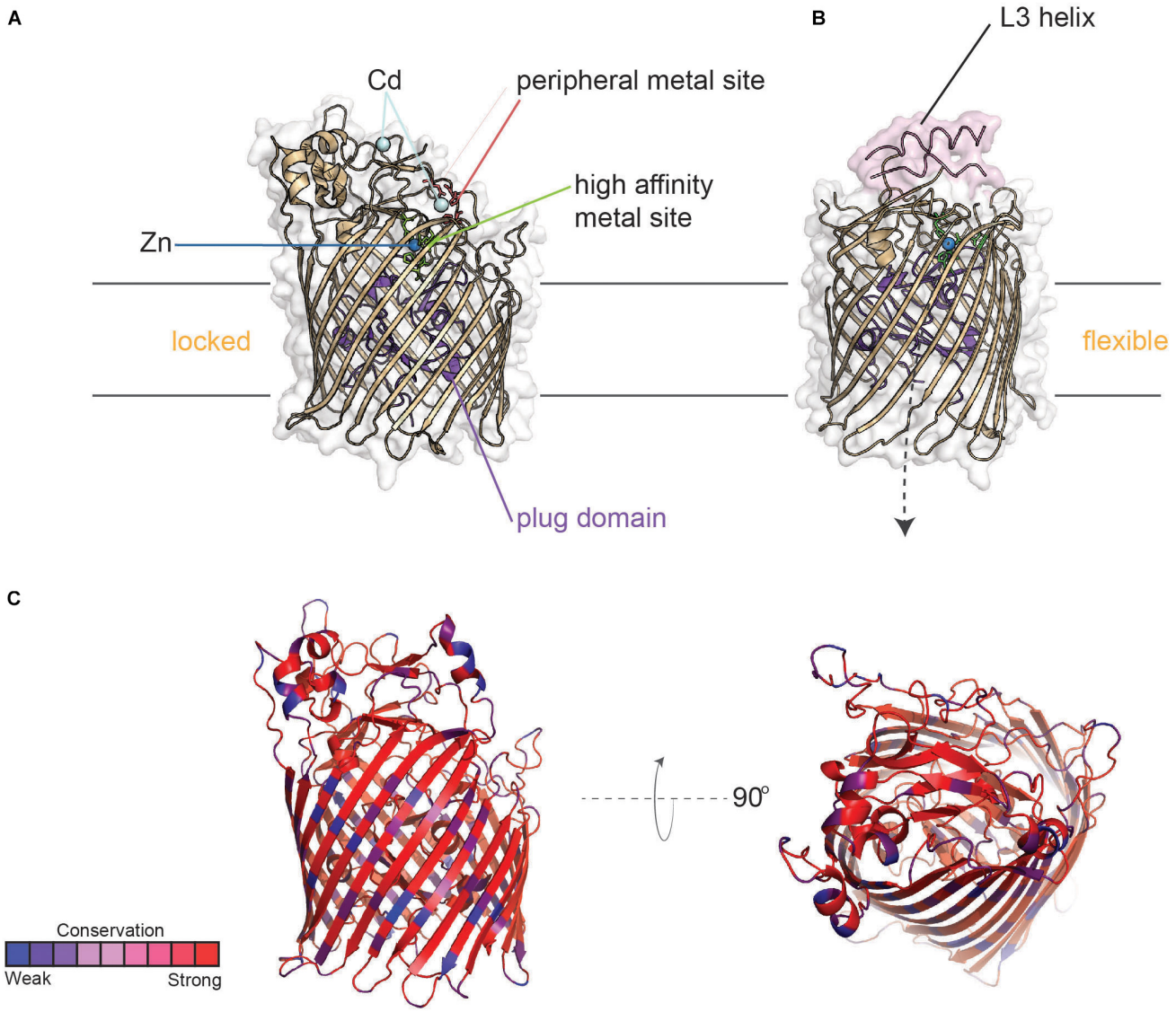

FIGURE 9 | Structure of TdfJ (ZnuD) in locked and flexible conformations. **(A)** Crystal structure of TdfJ (ZnuD) (PDB ID 4RDR) in the locked conformation. **(B)** Crystal structure of TdfJ (ZnuD) in the flexible conformation (PDB ID 4RDT). **(C)** TdfJ (ZnuD) with primary sequence conservation scores from AL2CO mapped onto the tertiary structure for 78 distinct (90% sequence identity cutoff threshold) *N. meningitidis* isolates from BigsDB (also see **Supplementary Figure 7**).

from *N. meningitidis* shows that extracellular loop 3 contains an exposed helix similar to that of TbpA which coordinates transferrin utilization. Strikingly, ZnuD loop 3 is flanked on each side by a His/Asp-rich region that senses zinc (Calmettes et al., 2015), so it is possible that this helix finger in TdfJ may play a role in the interaction with S100A7, but this has not yet been tested. Interestingly, ZnuD undergoes a significant conformational change upon zinc binding. By remodeling the external loop structures, ZnuD goes from a "locked" position (**Figure 9A**) to a more "flexible" state upon zinc binding (**Figure 9B**), allowing for metal sequestration in a buried high-affinity site (Calmettes et al., 2015). Like the other TdTs discussed above, TdfJ (ZnuD) is well conserved, but has some sequence variation strain-to-strain within the *Neisseria* species (**Figure 9C**). TdfJ is also not subject to high-frequency phase or antigenic variation. As seen with the Tbps and Lbps, it is possible that the two aforementioned zinc acquisition systems create redundancy, but experimentation is needed to test for any compensatory effects.

CONCLUSION AND PERSPECTIVES

Neisseria gonorrhoeae is an urgent threat pathogen that is evolving resistance factors faster than therapeutic interventions are being developed. There is currently no preventative vaccine for this STI and infections are not protective against subsequent exposures. Thus, new therapies and prevention strategies are urgently needed against this very common pathogen. While

N. meningitidis treatment and prevention are considerably more straightforward and well-developed, recent evidence suggests that vaccines to prevent meningococcal infections could also confer some level of protection against gonorrhea. Given that the newer-generation vaccines against *N. meningitidis* contain outer membrane vesicles and highly conserved outer membrane proteins, this cross-protection is perhaps not surprising. This proof-of-principle however, further suggests that deploying conserved outer membrane proteins and transporters as part of an anti-gonococcal vaccine could further protect against meningococcal infections as well. While the importance of expression of the transferrin binding proteins has been demonstrated in a human infection model, none of the other outer membrane transporters have been tested for their impacts on virulence in humans. Animal modeling is limited due to the species restriction of binding only to the human proteins. However, given the clear-cut advantage that the transferrin-iron acquisition system confers on wild-type *N. gonorrhoeae*, it seems likely that these other metal transport systems, which similarly enable the pathogen to overcome nutritional immunity, are equally critical to virulence. Interference with multiple metal uptake systems is likely to reduce the possible redundancy among the systems and hopefully result in greater killing by overpowering the pathogen's ability to subvert nutritional immunity. While current efforts are largely focused on vaccine development against *N. gonorrhoeae*, the same approach can be directed at identifying targets for newer-generation antibacterial drugs. Targeting one or more outer membrane transport systems that are responsible for importing necessary nutrients into the cell could be an Achilles heel for this stealth pathogen. Such an approach will require detailed structures of both transporter and bound human proteins, the origins of which are described in this review.

AUTHOR CONTRIBUTIONS

CC, NN, and TM initiated the collaborative project and acquired funding to support the project. RY and NO contributed the images and participated in writing the manuscript. TM, JS, SM, and CC contributed to writing the manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Progress Toward a Gonococcal Vaccine: The Way Forward

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The concept of immunizing against gonorrhea has received renewed interest because of the recent emergence of strains of *Neisseria gonorrhoeae* that are resistant to most currently available antibiotics, an occurrence that threatens to render gonorrhea untreatable. However, despite efforts over many decades, no vaccine has yet been successfully developed for human use, leading to pessimism over whether this goal was actually attainable. Several factors have contributed to this situation, including extensive variation of the expression and specificity of many of the gonococcal surface antigens, and the ability of *N. gonorrhoeae* to resist destruction by complement and other innate immune defense mechanisms. The natural host restriction of *N. gonorrhoeae* for humans, coupled with the absence of any definable state of immunity arising from an episode of gonorrhea, have also complicated efforts to study gonococcal pathogenesis and the host's immune responses. However, recent findings have elucidated how the gonococcus exploits and manipulates the host's immune system for its own benefit, utilizing human-specific receptors for attachment to and invasion of tissues, and subverting adaptive immune responses that might otherwise be capable of eliminating it. While no single experimental model is capable of providing all the answers, experiments utilizing human cells and tissues *in vitro*, various *in vivo* animal models, including genetically modified strains of mice, and both experimental and observational human clinical studies, have combined to yield important new insight into the immuno-pathogenesis of gonococcal infection. In turn, these have now led to novel approaches for the development of a gonococcal vaccine. Ongoing investigations utilizing all available tools are now poised to make the development of an effective human vaccine against gonorrhea an achievable goal within a foreseeable time-frame.

Keywords: ***Neisseria gonorrhoeae*, immunity, vaccine, antigens, antibodies, T cells, cytokines**

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INTRODUCTION

The concept of vaccinating against gonorrhea dates back to the earliest years following the discovery of *Neisseria gonorrhoeae* as the causative agent of this sexually transmitted disease in the late nineteenth century. In the early twentieth century, numerous attempts were made to treat gonorrhea by injecting various whole cell vaccines in the belief that these would promote opsonophagocytosis (1), which had been just been discovered by Wright and Douglas (2). However, none of these approaches were successful, and most were poorly controlled and inadequately described efforts. The advent of chemotherapy, first with sulfonamides (1936) and

soon followed by penicillin (1943), afforded dramatically successful treatment, obviating the need for vaccine development. However, resistance to these antibiotics soon emerged, a pattern repeated as each subsequent antibiotic was introduced, until the present when current U.S. guidelines call for dual treatment with a cephalosporin plus azithromycin (3). Unfortunately, resistance to both of these drugs is now emerging and several instances of treatment failure to such combinations have now been reported (4). As a result, authorities such as the U.S. Centers for Disease Control and Prevention and the World Health Organization have called for renewed efforts at gonococcal vaccine development.

An outside observer might reasonably ask why we do not already have a vaccine against gonorrhea, given that *N. gonorrhoeae* has been known as the causative agent for such a long time. However, a combination of three major factors has contributed to this situation. First, like a number of other infections, an episode of gonorrhea does not confer protective immunity against repeat infection, which is a relatively common occurrence. Consequently, in the absence of a clear state of immunity to gonorrhea in humans, it has not been possible to define the determinants or even correlates of immunity. Secondly, gonorrhea is a uniquely human disease, and *N. gonorrhoeae* has no known natural hosts other than humans. Thus, despite various efforts, it has been difficult to establish an animal model of infection, especially one that would replicate the human disease, in which immune responses and vaccines can be evaluated. As will be discussed below, this situation has been rectified, at least in part, by the development of a female mouse model (5), which has now been used in several laboratories to reveal unexpected aspects of immunity to *N. gonorrhoeae*. Finally, *N. gonorrhoeae* has long been known to display highly variable antigenicity, such that most of its main surface antigens continually evolve their sequence and/or reversibly phase-vary their expression on and off. While many pathogens utilize antigenic variation as a strategy of immune evasion, few do so to the extent that *N. gonorrhoeae* displays. As outlined below, elegant studies over the past three decades have revealed several mechanisms that independently promote this antigenic variation. This genetic plasticity complicates the assessment of specific immune responses to infection since even the same isolate will be antigenically different upon repeated passage. In addition, many gonococcal antigens are similar to those found in other neisserial species, including the closely related human pathogen *Neisseria meningitidis* and a variety of commensal *Neisseria* species commonly found in the human mouth and pharynx. Thus, most adults display serum anti-gonococcal antibodies regardless of whether or not they have been exposed to *N. gonorrhoeae*. As a result, it has proven impossible to define serological criteria—antibody levels—that unequivocally indicate previous exposure to *N. gonorrhoeae*, or that might indicate protective immunity.

PHASE AND ANTIGENIC VARIATION

Any consideration of gonococcal infection must take into account the unrivaled variability of this organism. *N. gonorrhoeae* is well-known for its extraordinary capacity to vary its surface antigen composition, both between strains and within the same strain over time. In the case of gonococcal type IV pili, promoterless copies of the genes that encode the major pilus subunit provide a silent repository of sequences that can be shuffled into an expression locus to generate new antigen variants. Aside from this intra-chromosomal recombination, *N. gonorrhoeae* is also highly competent for genetic transformation, allowing it to sample DNA fragments present in the mucosal environment in which it exists. This is facilitated by two different processes: a type IV secretion system that actively pumps single-stranded DNA into the environment (6) and a DNA uptake system that effectively binds and takes up DNA so that it may recombine into the chromosome if homologous sequences are present. This latter process is particularly efficient with DNA fragments containing short (∼10 base pair) non-palindromic conserved "uptake sequences" widespread throughout the neisserial genome (7, 8), which allow the bacteria to preferentially select DNA originating from pathogenic or commensal *Neisseria* species. This allows the rapid sharing of new genetic features, such as new resistance alleles when under antibiotic selection or variant alleles under adaptive immune pressure, and makes the genetic structure of the *N. gonorrhoeae* population panmictic rather than clonal.

Augmenting the effect of recombination-dependent antigen variation, the expression of many proteins is reversibly switched off and on through a process known as phase variation (9). The ongoing phase and antigenic variation, occurring independently in each bacterial cell, has the effect of continuously diversifying the population with respect to the surface epitopes that they express and, in many cases, the phenotype of the bacteria. It is, therefore, a combination of immune-based selection and the fitness conferred by each combination of attributes expressed that determines which variants dominate the population. The genetic processes that mediate neisserial genetic plasticity are the focus of an excellent recent review (10). Herein, we will briefly consider several different surface antigens that have been considered as vaccine targets to illustrate how each of these processes are accomplished, how they contribute to infection and immune evasion, and why they must be considered in vaccine development.

Porin

The porin, PorB, is the most abundant outer-membrane protein and is constitutively expressed by *N. gonorrhoeae*. Each isolate possesses a single *porB* allele, which encodes an outer membrane-expressed β-barrel with 16 membrane-spanning sequences and 8 surface-expressed loops (11). Targeting PorB in a vaccine is confounded by extensive variation within the sequence and length of its surface-exposed loops expressed by different strains, which provides the basis for gonococcal serogroup and serovar typing schemes (12). Aside from their antigenic differences, *porB* alleles can differentially contribute to processes including serum complement resistance (13, 14), apoptosis (15–17), and host cellular invasion (18, 19). In addition, properly folded PorB, as found in its native outer membrane environment, has a potential role in suppressing the adaptive immune response by inhibiting dendritic cell stimulation of CD4+ T cell proliferation (20). While the *porB* allele expressed is considered

to be a relatively stable phenotype within a strain, genetic transformation and recombination between co-infecting strains generates a PorB mosaicism that results in variants with new antigenic and, presumably, functional characteristics (21). This poses a challenge with regards to targeting the antigen; however, PorB has a potent TLR2-dependent adjuvant effect that stems from its tendency to form self-aggregating micellular structures, suggesting that it may be an interesting vaccine component (22).

LOS Glycan Structure

While phase variation most obviously alters protein expression, the differential expression of enzymes can also alter glycan epitopes on the gonococcal surface. The glycan side-chains of the lipo-oligosaccharide (LOS) are determined by cytosolic glycosyltransferases, which are subject to phase-variable expression (23, 24). This process occurs through a slip-strand mispairing of homopolymeric nucleotide tracts within the regions encoding LOS glycosyltransferase genes, which leads to addition or subtraction of the repeated oligonucleotide sequences and, thereby, expression or not of the enzyme based upon whether its coding sequence is in or out of the translation reading frame (23, 25–27). Aside from influencing immune cross-reactivity between isolates, LOS variation can also directly impact gonococcal association with epithelial cells (28) and the recruitment of serum complement regulatory proteins that protect against the bactericidal activity of blood (29, 30). A monoclonal antibody, 2C7, generated from a mouse immunized with gonococcal outer membranes, recognizes a lactose residue substituted on heptose I or heptose II of gonococcal LOS (31). This antibody also reacts with 95% of fresh clinical isolates (31), and it enhances clearance of *N. gonorrhoeae* infection in a mouse lower genital tract infection model (32), suggesting that such phase-variable antigens might be effective vaccine targets if there is sufficient selection for their expression during natural infection. However, the 2C7 epitope can be lost due to phase-variable expression of LOS glycosyltransferases (33).

Opa Proteins

The gonococcal colony opacity-associated (Opa) proteins are a large family of integral outer membrane proteins each consisting of a β-barrel with 8 highly conserved transmembrane sequences that display four surface-exposed variable loops (34). A single gonococcal isolate possesses ∼11 different *opa* genes, the expression of each being independently and stochastically regulated by phase variation such that the bacteria may express no Opa proteins or any combination of one or more Opa variants. This on-off switching occurs because, although each locus is constitutively transcribed at a high level, the protein-coding sequence can "slip" in or out of the translational reading frame due to the addition or loss of one or more pentanucleotide (CTCTT) repeat sequences in the structural gene that encodes the amino-terminal leader peptide (35). As with other repeat sequences that lead to phase-variable gene expression, the addition or subtraction of pentanucleotide repeats can occur during chromosomal replication at cell division or by homologous recombination between *opa* loci, both of which contribute to frequent (10^{-3} per generation) changes in Opa phenotype. The large number of *opa* genes also allows ongoing intra-chromosomal and horizontal (inter-strain) recombination between the conserved membrane-spanning or periplasmic sequences, resulting in the replacement of one or more surface loops (36); this explains the hundreds of *opa* alleles described to date. Remarkably in the context of this variability, most Opa variants retain protein-protein binding to human carcinoembryonic antigen-related cellular adhesion molecules (CEACAMs), their human cellular receptors (37–43), and retain their lectin-like function that mediates inter-bacterial aggregation (44). This conservation of function allows persistent expression of these binding phenotypes while allowing the bacteria to evade Opa variant-specific immunity.

Pilus

The type IV pili are, perhaps, the most remarkable structures on the gonococcal surface, extending ∼1 μm from the outer membrane to allow human cellular attachment before retracting to allow tight apposition of the bacterial and host cell surfaces. The pilus fiber is composed of a helical array of pilin proteins, the preponderance being the major pilin protein, PilE (45, 46). Aside from the full length *pilE* expression locus, there are ∼20 transcriptionally "silent" *pilS* loci (47, 48). These are effectively repositories of variant pilin sequences because each encodes the surface-exposed variable regions interspersed by conserved sequences that make up the pilin protein core, but have no promoter sequence or start codon. Rather than being mediated by simple homologous recombination, this process involves a "gene conversion" event that allows alteration of the target expression locus while also retaining the variable sequences within the "donor" *pilS* locus (49). This ongoing high-frequency variation assures that the bacteria display an ever-changing array of epitopes that makes it impossible for the adaptive immune response to catch up to.

The remarkable ability of *N. gonorrhoeae* to generate antigenic variation while retaining functionality of its surface proteins has engendered the belief that, while an antibody response might be induced by infection, this would be rendered ineffective as subpopulations of bacteria are always present that can avoid antibody recognition. This, along with more recent findings concerning gonococcal immune evasion, has led to fears that it might not be possible to elicit immune protection. However, as discussed below, evidence is now accumulating that, while *N. gonorrhoeae* possesses the ability to subvert the development of an effective immune response, an appropriately targeted *N. gonorrhoeae*-specific immune response can be protective.

PREVIOUS VACCINE EFFORTS

Early work with chimpanzees provided the first indication that gonococcus-specific immunity can be achieved. Experimentally inoculated male chimpanzees were observed to clear urethral infection within several weeks, and a 3-log higher infectious dose was required to re-infect the animals 1 week after the infection had cleared. These animals were not immune when re-exposed 2 years after the primary infection, suggesting that this immunity

waned over time (50). Subsequent studies with a killed whole-cell gonococcal vaccine reduced susceptibility to gonococcal infection, as indicated by the requirement for an increased infectious dose (51), suggesting that parenteral immunization might be a reasonable approach.

Only two vaccine candidates have been tested in field trials with humans. The first was a parenterally delivered heat-killed, partially lysed whole-cell vaccine that induced serum antibody titers in 90% of subjects but showed no protection based upon a similar number of individuals infected within 12 months after vaccination (52, 53). The second gonorrhea vaccine tested in humans was a purified pilin vaccine that was delivered intradermally using a two-dose regimen separated by 2 weeks to over 3,000 US military personnel in Korea. No difference was found between groups given the vaccine vs. a placebo, with 108 and 102 subjects, respectively, acquiring gonorrhea 2 weeks after inoculation (54). The likelihood that antigenic variability of gonococcal pili was responsible for, or at least contributed to, the failed study is supported by reports that this vaccine showed efficacy during in-house trials using the homologous strain (55) but not a heterologous strain (56). Notably, pilus-specific mucosal antibodies that blocked gonococcal adherence to human cells appeared to be a correlate of protection in this trial (57), providing perhaps the clearest indication of how immunity might be achieved. Regardless, the point that efficacy was observed in the homologous challenge trial should not be ignored since it counters the often-held assumption that immunity to *N. gonorrhoeae* was not achievable in humans.

The momentum toward developing a gonococcal vaccine was lost with onset of the HIV epidemic in the early 1980s, which diverted investigators and research funding away from gonorrhea and other STDs. The focus on HIV also meant that chimpanzees were no longer available for gonococcal vaccine studies, and there was not yet a small animal model for systematic testing of candidate vaccines and the immune responses they elicit. These events, when combined with the failure of the first human trials, had the unfortunate effect of reducing the interest of the pharmaceutical industry and other agencies, and of discouraging further gonococcal vaccine research for decades. With recent increases in the global burden of gonococcal infection and the emergence of multidrug resistant strains, this situation has now begun to turn around.

NATURAL INFECTION DOES NOT INDUCE PROTECTIVE IMMUNITY: STUDIES WITH HUMANS

Studies in humans have been complicated by numerous factors including: logistical difficulties in recruiting volunteers willing to adhere to study protocols; practical considerations of defining matched infected and control groups to account for confounding factors; the frequent occurrence of "asymptomatic" infections in both females and males, which creates uncertainty over the time elapsed between exposure and diagnosis of infection; different states of infection in males and females, ranging from uncomplicated urethritis or cervicitis to complicated upper tract infection (prostatitis or epididymitis in males, endometritis, and salpingitis with pelvic inflammatory disease in females) and, more rarely, disseminated systemic infection; the frequent occurrence of co-infections; the unreliability of subjects' self-reporting previous infection, which is further confounded by asymptomatic infection; and the availability of funds. Ethical considerations also impose constraints because subjects presenting at clinics must obviously be offered treatment upon diagnosis, thereby limiting longitudinal studies aimed at understanding the natural progression of infection and immunity. Experimental studies involving challenge infections are limited to males because of the risk of complications and sequelae in females. However, even in males, the requirement to treat the infection shortly after symptoms develop means that only short-term studies focused on the establishment of infection are realistically possible.

Despite the challenges associated with studying infection in humans, numerous studies have attempted to comprehend the interaction of *N. gonorrhoeae* with various components of the human immune system. From these, certain conclusions can be drawn, understanding of course that the relative contribution of these effects during natural gonococcal infection remains poorly understood.

Antibody and cell-mediated (cytokine) responses to documented, uncomplicated gonococcal infection in both women and men are weak and of short duration (58, 59). Assays of antibody and T cell responses are complicated by the extensive antigenic variability of *N. gonorrhoeae*, which means that it is difficult to define a standard antigen preparation against which to evaluate responses. However, even when responses were evaluated against homologous gonococcal isolates, it became clear that antigenic variation does not explain the absence of response since reactivity with the homologous strain was little more than that measured against a prototypical laboratory-adapted strain (58). These observations, coupled with the finding that responses tended to dissipate after a few weeks, and were apparently no greater in individuals documented to have had previous infections, led to the proposition that *N. gonorrhoeae* was somehow capable of interfering with the normal course of an adaptive immune response (59). In contrast, men experimentally infected with *N. gonorrhoeae* in the urethra were found to show elevated levels of inflammatory cytokines (IL-8, IL-6, IL-1β, and TNF) in plasma and urine (60). In a subsequent study on experimentally infected men, increased titers of anti-LOS IgG antibodies were observed in some subjects, but no evidence was seen for enhanced resistance to re-infection with the same gonococcal strain 2 weeks after treatment of the initial infection, compared to previously uninfected controls (61).

The lack of specific protective immunity stands in marked contrast to the strong inflammatory response elicited during symptomatic infection, which is evident by the massive influx of neutrophils, as well as by the inflammatory cytokine responses noted above. However, insufficient attention has been given to the different states of infection in men and women, and the possibility that host adaptive responses might accordingly be different. Indeed, the recognition that women who suffer from severe cases of PID have heightened gonococcal-specific

antibodies and antibody-dependent serum bactericidal activity (62), as well as protection against salpingitis (63), clearly demonstrates that context is important. Given that systemic gonococcal infection can also elicit a memory immune response (64), it seems reasonable to consider that an adaptive response might depend on gonococcal tissue penetration during these more severe manifestations of infection. This requirement would explain why most gonococcal lower urogenital tract, pharyngeal, or rectal infections, which are either asymptomatic or induce little tissue damage, do not elicit a memory response. Nevertheless, when considered together, these findings collectively support the hypothesis that *N. gonorrhoeae* can interfere with the immune response to uncomplicated infection at the mucosal surfaces.

Humoral Immunity

Whether gonococcus-specific antibodies are present or not, *N. gonorrhoeae* has been shown to be capable of obviating complement-dependent bacteriolysis, and several mechanisms have been described. Gonococcal sialylation of its LOS by means of its surface-expressed sialyl-transferase (65), which utilizes host-derived cytidine monophosphoryl-*N*-acetyl-neuraminic acid (CMP-NANA) as substrate, inhibits complement (C3) deposition to facilitate gonococcal survival in human blood (66, 67) and in mice (68, 69). Sialylation of LOS also enhances binding of human complement regulatory factor H to PorB (70). Some naturally occurring variants of gonococcal PorB can interfere with the alternative and classical pathways of complement activation by binding to factor H or C4b-binding protein (C4BP), respectively (71, 72). It is important to note that PorB binding is specific for human C4BP and factor H, so these effects may not be evident in serum bactericidal assays if non-human complement is used or in most animal infection models. Gonococcal NspA (neisserial surface protein A) also binds human factor H (73), but the gonococcal homolog of the meningococcal factor H-binding protein lacks a signal peptide and therefore is not available on the cell surface to bind factor H (74).

While the mechanism remains poorly understood, the highly immunogenic reduction-modifiable protein (Rmp), which sits closely associated with porin on the gonococcal cell surface, elicits antibodies that block complement activation by IgG antibodies against LOS or porin (75). This mechanism, which serves to enhance gonococcal survival in mice as well as in human serum (76), has led to the hypothesis that protective immunity can be defined by the ratio of antibodies to LOS and porin over antibodies to Rmp (76). However, in the absence of a state of immunity to gonococcal infection in humans, this hypothesis has not been confirmed or refuted.

Finally, based upon parallels with studies done with *N. meningitidis*, it appears likely that the gonococcal IgA1 protease also confers a protective effect. Specifically, IgA antibodies specific for the meningococcal capsular polysaccharide can block complement-mediated bacteriolysis caused by IgM or IgG antibodies to the same antigen (77). In this case, the Fabα fragments generated upon cleavage of IgA1 antibodies by the meningococcal IgA1 protease mediate this effect through their ability to mask epitopes and, thereby, prevent binding of functional full length (Fc-containing) antibodies (78, 79). As all strains of *N. gonorrhoeae* constitutively produce a homologous IgA1 protease (80), it seems likely that a similar mechanism operates with gonococcal infection, but evidence in support of this has not been forthcoming (81). Additionally, it has been proposed that gonococcal IgA1 protease enhances intracellular survival by cleaving the lysosomal protein, LAMP1 (82, 83), suggesting that this virulence factor has multiple infection-enhancing effects.

Interactions With Phagocytes

The hallmark of gonorrhea has long been the presence of a purulent discharge that consists primarily of neutrophils, many of which are associated with apparently intact diplococci. It is well-known that phagocytic uptake and killing of bacteria is greatly enhanced when the bacteria are coated with opsonins consisting of antibodies and/or complement. The "professional" phagocytes, i.e., neutrophils and macrophages, express receptors for the Fc regions of IgG (FcγRs) and IgA (FcαRI), which carry signaling immuno-tyrosine activation motifs (ITAMs) on their cytoplasmic domains, and for C3b. These receptors not only promote attachment of bacteria to the phagocytes, but the Fc receptors also stimulate intracellular signaling events that result in release of bactericidal granules into the phagocytic vesicles and trigger the oxidative burst that generates reactive oxygen species, thereby leading to killing of ingested bacteria. However, the interaction of *N. gonorrhoeae* with human neutrophils is not necessarily dependent on opsonization, since it can also be mediated by gonococcal pili and Opa protein adhesins binding to their host cellular receptors, especially during the early stages of infection. Gonococcal variants lacking these adhesins are less readily ingested and killed by neutrophils (84, 85). In later stages when inflammation has been induced with exudation of plasma proteins, the increased availability of complement components, especially C3, allows the binding of C3b to the gonococcal surface by alternative pathway activation (86) or even direct proteolytic cleavage of C3, and subsequent binding to C3b receptors on neutrophils.

The adhesin-mediated interaction with phagocytes is host-restricted, since non-opsonized gonococci avoid capture by mouse neutrophils regardless of whether pilus or Opa proteins are expressed. However, gonococci are effectively engulfed if transgenic mouse neutrophils express human CEACAMs that function as the cellular receptors for the neisserial Opa proteins (87, 88). Particularly notable in this regard, CEACAM3 is an innate decoy receptor expressed exclusively on human neutrophils. The CEACAM3 extracellular domain mimics epithelial cell-expressed CEACAMs, allowing effective gonococcal binding. Because its cytoplasmic domain carries a signaling ITAM motif similar to those on Fc receptors, Opa protein-dependent engagement of CEACAM3 triggers bacterial phagocytosis (89–93), neutrophil degranulation and oxidative burst (40, 90, 91), as well as a pro-inflammatory chemokine response that drives ongoing neutrophil recruitment to the infected tissues (87, 88, 94). Depending upon the

bacterial burden and presumably other factors, this CEACAM3-dependent response can either effectively clear the infection or contribute to the self-propagating inflammation that typifies gonococcal disease. However, the phase-variable expression of Opa proteins, some of which do not recognize CEACAMs, always maintains some gonococci that evade capture by neutrophils (43, 95, 96) and, thereby, avoid this bactericidal response (43, 97–99). These opposing effects drive *in vivo* selection for Opa variants that do not engage CEACAM3 but still allow epithelial cell attachment (42).

Opsonin-dependent uptake of gonococci by phagocytes is limited by the paucity of IgG (and IgA) antibodies, the production of which is suppressed by *N. gonorrhoeae* (as discussed below) and rendered ineffective by antigenic variation, and by several gonococcal mechanisms that inhibit complement activation or deposition. For example, sialylation of LOS by means of host-derived CMP-NANA inhibits C3b deposition on the gonococcal surface, thereby diminishing phagocytic uptake (68, 100, 101). While the gonococci are usually sialylated during infection, most *in vitro* phagocytosis experiments have not utilized *N. gonorrhoeae* grown in the presence of CMP-NANA to ensure that it is sialylated, which may result in misleading conclusions. In addition, *N. gonorrhoeae* resists intracellular phagocytic killing in several ways, including suppression of the oxidative burst, resistance to non-oxidative killing mechanisms, and the production of lytic transglycosylases (102–106). The extent to which opsonization of *N. gonorrhoeae* by specific IgG (or IgA) antibodies and/or complement can overcome its ability to avoid intracellular killing requires further investigation.

While largely overlooked due to the striking role that neutrophils play in gonorrhea, recent work has begun to illuminate the role that other phagocytes play during infection. Gonococcal infection of primary macrophages promotes their survival and stimulates their expression of pro-inflammatory cytokines (107), suggesting that these innate cells may play an important role in the recruitment of neutrophils during the early stages of infection. However, exposure to *N. gonorrhoeae* promotes human monocyte differentiation into macrophages with a tissue repair (M2) phenotype that are unable to stimulate T cell proliferative responses (108, 109). The relative contributions of inflammatory and inhibitory macrophage responses might influence the intensity of the overall response and thereby affect the outcome of gonococcal infection.

A mechanistic explanation for the suppressive effect of *N. gonorrhoeae* on macrophages remains to be determined. However, it at least functionally resembles that seen with human monocyte-derived dendritic cells, where Opa protein-dependent binding to the immune co-inhibitory receptor CEACAM1 suppresses their normal maturation into antigen-presenting cells (110). This, when coupled with the fact that Opa binding to CEACAM1 on CD4+ T cells effectively inhibits their ability to be stimulated by T cell receptor engagement or cytokine exposure (111, 112), provides a clear indication that gonococci have the capacity to directly interfere with the adaptive response in humans. However, rather than simply suppressing immunity, gonococci also appear to misguide the response by virtue of their ability to drive a robust T cell-independent activation of the IgD- and CD27-expressing subset of innate-like human B cells, which results in the rapid accumulation of broadly-reactive (not *Neisseria*-specific) low affinity IgM antibodies (113). It is enticing to consider that this effect might promote a localized increase in irrelevant antibodies during human genital infection with *N. gonorrhoeae*, an effect that would both be ineffective and have a suppressive effect on other B cells.

ANIMAL MODELS

The classic paradigm, that vaccination seeks to mimic a natural infection to induce immunity without causing disease, is clearly inapplicable to gonorrhea because the essential premise, that natural infection induces immunity, is not true. Instead, it becomes necessary to comprehend how a reactogenic inflammatory infection such as gonorrhea interacts with the immune system so as to avoid inducing an effective response. An effective vaccine will need to avoid this same outcome. However, experimental investigation of gonococcal pathogenesis and the host response, and understanding of the factors that affect these responses, have been severely limited by strict human specificity of *N. gonorrhoeae*, which challenges animal modeling of this infection. Ethical considerations prohibit the study of the human disease in the absence of treatment, and various efforts to infect other animal species in a manner resembling the human disease have been unsuccessful (114). A possible exception is the reported genital infection of a male chimpanzee and the subsequent sexual transmission of infection to a female (115), but experimenting on this species would be prohibitively expensive and, in any case, is now impermissible. Without a protection model, vaccine development has been stunted because efficacy testing has not been possible. Fortunately, this situation has been partially rectified by the development of the estradiol-treated female mouse model of genital tract gonococcal infection (5), which is robust, genetically tractable, and has been extensively used in various laboratories over the past two decades.

Inbred Mouse Strains

In the 1980s it was reported that female mice can be transiently colonized with *N. gonorrhoeae* if inoculated in the proestrus stage of the estrous cycle, when vaginal neutrophils and commensal flora are low (116–118). Colonization cleared, however, upon transition to the late estrus/metestrus stages. These observations led to the demonstration that germ-free BALB/c mice treated with 17-β-estradiol could be vaginally colonized with *N. gonorrhoeae* for several weeks, whereas mice that were instead treated with progesterone resisted infection (119). This model was made more broadly practical by subsequent work demonstrating that conventional (not germ-free) 17-β-estradiol-treated BALB/c mice could be infected if estrogen-induced overgrowth of their vaginal microbiota were suppressed by antibiotic treatment (5). It seems likely that the high numbers of neutrophils present in the vaginal lumen during the progesterone-dominant diestrus stage explain the resistance of progesterone-treated mice to gonococcal infection, while the bloom in commensal microbes that normally occurs due to increased glycogen secretion by vaginal epithelium during

estrus demands the use of antibiotics in estradiol-treated mice. However, given that the sex hormones can influence immune cell function (120, 121), as well as epithelial expression of innate inflammatory mediators (122, 123) and genital tissue architecture (124), other factors undoubtedly contribute to the success of this approach. Nonetheless, the impact of female hormonal cycling on gonococcal infection in mice (125) reflects clinical observations that gonococcal abundance also appears to increase during the proliferative (high estrogen) stage and then wane during the luteal (high progesterone) stage of the menstrual cycle in women (126, 127). Likewise, there are broad similarities in vaginal carbon (lactate and glucose) availability (128), oxygen tension, physicochemical properties of mucus, and interactions with commensal microbiota (129–131). However, pH differs: mouse vaginal pH ∼6.2, human vaginal pH ∼4.0, although human cervical pH ∼6.0 (130). Iron restriction differs in that *N. gonorrhoeae* can extract iron from human transferrin and (in some strains) lactoferrin, whereas it is unable to utilize murine transferrin or lactoferrin (132). Nevertheless, the mouse infection model affords a valuable surrogate for evaluating both innate and adaptive immune responses that cannot be adequately modeled *in vitro*, and is currently the only available model in which the interaction of *N. gonorrhoeae* and an intact mammalian immune system can be studied. **Table 1** summarizes the similarities and differences between gonococcal infection in humans and mice.

While the BALB/c infection model is the most well-established, certain other (but not all) mouse strains can be similarly infected (5, 133). Notable in this regard is that C57BL/6 and BALB/c mice are similarly permissive to infection, but the former do not reveal the neutrophil response shown by the latter (133), perhaps suggesting that a marked neutrophil response does not greatly influence the course of infection in mice. While it is also tempting to contemplate that the relative susceptibility of each inbred strain is determined by varying innate or adaptive immune responses stemming from their genetic differences, it is also possible that susceptibility is related to differences in the genital microbiota carried by the mice, which in part also varies with their source. This is particularly interesting considering that *Lactobacillus*-derived lactate is a preferred carbon source for *N. gonorrhoeae* (128), that components of the microbiota can influence gonococcal growth *in vitro* (129, 134), and that microbe-derived sialidases can modify the gonococcal LOS to facilitate its association with epithelial cells (135).

Genetic Manipulation of Mice

Aside from the obvious benefit of being able to experimentally infect mice with naturally occurring or recombinant gonococcal strains, the murine system also allows genetic manipulation of the host. This has been particularly useful for efforts to understand determinants of immunity. For example, isogenic mouse lines lacking the endotoxin receptor, Toll-like receptor 4 (TLR4), have a substantially higher burden of *N. gonorrhoeae* than do wild-type controls after vaginal infection, and this correlates with an exaggerated classical (TNF, CXCL1/KC, and CXCL2/MIP-2) inflammatory response but a diminished IL-17 response in TLR4-deficient mice (136–138). While it might be expected that innate recognition of microbe-associated molecular pattern (MAMP) molecules such as LOS would promote immune defense, these findings provide important mechanistic context for an emerging recognition that Th17-driven innate responses to gonococcal infection occur concomitantly with suppression of Th1/Th2-driven adaptive responses (138–140), which is discussed below. This disconnect between innate and adaptive immunity is fundamental to understanding why *N. gonorrhoeae* is such a successful human parasite, and exemplifies how mouse models provide important insight that could not emerge from other experimental approaches.

When modeling any infection, whether using *in vitro* cellular infection or a non-natural host, it is imperative that any results be considered in the context in which they are being observed. Mouse infection models provide an opportunity to consider infection within a physiologically relevant mucosal environment, including a fully functioning immune system, conditions which are impossible to replicate outside of an animal. However, the fact that many gonococcal virulence factors only bind to their human-derived targets means that their contribution to infection cannot be appreciated in a wild type mouse. Given that the first step in infection is anchorage to host tissues, the fact that gonococcal pilus and Opa protein adhesins do not bind mouse tissues provides the most obvious example of this point. The elegant means by which *N. gonorrhoeae* overcomes nutritional immunity, including both iron (141, 142) and zinc (143) acquisition systems, and by which it binds to human proteins to evade serum complement-dependent killing (144, 145), are rendered ineffective in the mouse.

Fortunately, the genetic tractability of mice has allowed direct investigation of the impact that well-characterized gonococcal effector proteins play *in vivo*. This strategy has recently been applied for the generation of transgenic mice expressing different combinations of human CEACAMs, which are the receptors for most neisserial Opa protein adhesins. While an enormous amount of primary and immortalized human cell-based work has described how individual human CEACAMs can each contribute to the outcome of gonococcal interactions with different cell types (37–41, 89–91, 93, 99, 110, 111, 146–154), the gonococcal Opa proteins do not bind mouse CEACAM1 and mice do not express CEACAM3, CEACAM5, or CEACAM6, so all four targets are effectively absent. Transgenic mice have now been generated to encode various combinations of these receptors, and each CEACAM appears to be expressed with a cellular distribution reflecting that in humans, including CEACAM1, CEACAM3, and CEACAM6 on neutrophils, CEACAM5 on vaginal epithelium, and CEACAM1 on the endometrium (88, 94). Consistent with *in vitro* cell-based studies, human CEACAM5 expression promotes an intimate Opa protein-dependent attachment to mucosal tissues and an extended duration of colonization within the female lower genital tract (94). In addition to the direct effect of Opa binding on epithelial attachment, this outcome is facilitated by decreased exfoliation of infected epithelial cells due to the CEACAM-dependent upregulation of β1 integrin-mediated binding to their underlying extracellular matrix (155). However, the effect of CEACAM expression depends on the exposed tissue and stage of the female reproductive cycle since, for example, uterine inflammation occurs in transcervically

TABLE 1 | Similarities and differences in gonococcal infection between mice* and humans.

Aspect	Mouse	Human
Antibody response	None	Weak to none
Th1 response	No	No
Th2 response	No	No
Th17 response	Yes	Some evidence
Memory development	No	No
Protection against reinfection	No	No
Neutrophil influx	Depends on strain	Typical in symptomatic infection
Duration of infection	∼1–3 weeks	Several months?
Primary site of infection	Vagina and cervix	Cervix
Impact of reproductive hormones	Cyclical fluctuations in recovery of gonococci	Gonococcal recovery zero or low in luteal phase, increased in proliferative phase
Disease	Inapparent or inflammatory cervico-vaginal infection, depending on mouse strain. Ascending infection in ∼20% of mice following vaginal inoculation; endometrial infection in most mice following transcervical inoculation	Asymptomatic (m, f); Cervicitis (f), urethritis (m); Endometritis, salpingitis (f); Pelvic inflammatory disease (f); Prostatitis, epididymitis (m); Disseminated (m, f)

*f, female; m, male. *Male mouse model not available.*

infected wild-type mice in diestrus (156) but occurs only in the presence of human CEACAM1 when the mice are in estrus (94, 157). Moreover, the integration of CEACAM-dependent effects determines the outcome of infection since neutrophil expression of CEACAM3 promotes inflammation and phagocytic clearance of the gonococci, effectively opposing the infection-promoting contribution of epithelial CEACAMs (88, 94). What was not foreseen by *in vitro* studies was that the neutrophil response to CEACAM3 binding includes their triggering of a potent pro-inflammatory transcriptional program that stimulates an ongoing recruitment of neutrophils, an effect that may help drive the clinical manifestations of gonorrhea (43, 87, 88). Ongoing efforts to generate mice co-expressing human CEACAMs along with other factors targeted by neisserial virulence factors, including the human iron-sequestering proteins transferrin (141) and lactoferrin (142), zinc-sequestering calprotectin (143), and the complement regulatory proteins factor H (144) and C4 binding protein (145), will allow further appreciation of how these and other exquisite evolutionary adaptations contribute to the lifestyle of *N. gonorrhoeae* within human mucosal tissues.

Human Stem Cell-Repopulated Mice

Complementing the genetics-based approaches to understand infection, recent success in the engraftment of severely immunodeficient mice with a functional human immune system (158), raises the possibility that human-restricted immune cell responses might also be explored. Highlighting the potential impact of this approach, the female genital mucosa of NOD/*LtSz-scid/scidγ null* (NSG) mice that have been engrafted with human CD34+ hematopoietic stem cells becomes populated with human leukocytes (159). Given that they now produce human CD4+ T cells, these mice become susceptible to chronic HIV infection. Reflecting what is observed in humans (160, 161), the lower genital tract infection with *N. gonorrhoeae* promotes localized HIV shedding without impacting systemic viral titers (159). Thus, while technically difficult and too cost-prohibitive for routine studies, this approach provides an attractive model with which to study human-specific immune responses to gonococcal infection and for translational work aiming to promote immunity or to counteract gonococcal immuno-pathogenesis.

N. gonorrhoeae MANIPULATES THE IMMUNE RESPONSE

While early mouse infection studies aimed to understand how *N. gonorrhoeae* establishes infection, several groups have recently begun to use the model to discover how the gonococci subvert the immune response. Studies with experimentally infected female mice have shown that gonococcal infection of the genital tract leads to the production of the inflammatory Th17-associated cytokines, IL-17 and IL-22, but not specific antibodies or cytokines typical of Th1- or Th2-driven adaptive immune responses, such as IFNγ or IL-4 (137, 162). When IL-17 signaling was blocked by neutralizing antibodies or use of IL-17 receptor-deficient mice, the course of gonococcal infection was prolonged. These results indicate that innate defense mechanisms driven by IL-17, such as the recruitment of neutrophils and the secretion of anti-microbial proteins by epithelial cells, contribute to the elimination of the infection (137). Conversely, in IL-22-deficient mice, infection was more rapidly eliminated (163), implying that responses driven by IL-22 signaling promote gonococcal infection in this model. This is an unexpected result because this Th17-related cytokine tends to enhance epithelial defense by antimicrobial peptide expression and increased barrier function (164). The immune regulatory cytokine, TGFβ, was found to be important not only for the induction of the Th17 response, but also for the

suppression of Th1- and Th2-driven adaptive immune responses (138, 139). Notable in this regard, administration of TGFβ-neutralizing antibodies during gonococcal infection allowed the development of anti-gonococcal antibodies and the emergence of Th1 and Th2 cells secreting IFNγ and IL-4, respectively, which correlated with the establishment of immune memory and the accelerated clearance of infection. Furthermore, re-infection of mice treated with anti-TGFβ antibody during an initial (primary) infection resulted in recall of memory responses and enhanced resistance to re-infection (138). Subsequent studies found that gonococcal infection in mice also strongly enhanced the production of another regulatory cytokine, IL-10, and induced FoxP3-negative, IL-10-dependent, type 1 regulatory T (Tr1) cells (140). Neutralizing antibodies to IL-10, or the use of IL-10-deficient mice, showed that IL-10 contributed significantly to the suppression of adaptive immunity, and that its absence allowed protective antibody responses to develop. Conventional FoxP3-positive regulatory T cells have also been observed in mice infected with *N. gonorrhoeae* (165). Combined, these findings imply that *N. gonorrhoeae* selectively induces Th17-driven innate responses that it can resist, while concomitantly suppressing Th1- and Th2-driven adaptive immune responses that would eliminate it (166). It is satisfying that humans infected with gonorrhea have also been reported to show elevated serum IL-17 (and IL-23, which enhances Th17 development) (167, 168), although these studies do not definitively demonstrate that these cytokines were elevated in response to gonococcal infection.

In considering the translational implications of the aforementioned finding that *N. gonorrhoeae* induces a TGFβ- and IL-10-dependent suppression of adaptive immune responses, it seemed reasonable that IL-12, a cytokine known for its ability to antagonize IL-10 and to drive Th1 responses, should be able to reverse this induced suppression of adaptive immunity. Accordingly, local genital administration of IL-12 encapsulated in sustained-release microparticles during gonococcal infection induced Th1-dependent immune responses with the production of IFNγ by CD4+ T cells and anti-gonococcal antibodies, and accelerated clearance of the infection. Re-infection of mice that had received IL-12 during their prior infection revealed that they had become resistant to the challenge, with the recall of anti-gonococcal antibody responses as well as IFNγ (169). An unexpected finding was that resistance to re-infection was not limited to the strain used for the initial infection, but extended to other unrelated strains, including recent clinical isolates (170). While the extent of this immune cross-protection among different gonococcal strains remains to be evaluated, it suggests that immunity is likely not dependent on strain-variable antigens such as PorB, LOS, Opa proteins, or pilin. Furthermore, immunodeficient mice lacking either B cells (and hence the ability to generate antibodies) or IFNγ did not generate resistance to re-infection in response to treatment with microencapsulated IL-12 during the primary infection (170), implying that both antibodies and IFNγ were required for immunity. While a functional role for antibodies can be readily envisaged, for example in enhancing complement-dependent bacteriolysis or phagocytic killing, or by inhibiting epithelial colonization, the contribution of IFNγ remains unclear. However, IFNγ is known to promote B cell switching to the generation of Ig isotypes that would be most efficient in these effector functions (171–173), and to upregulate the activity of phagocytes such as neutrophils and macrophages (174, 175), making these plausible determinants of protection.

While these studies provide a satisfactory explanation as to why there is no adaptive response to infection (at least within the female mouse genital tract), the underlying mechanism remains unknown. For example, it remains unclear whether the Th17-dominated response is unique to the genital tract or is instead a specific effect of gonococcal infection. If the latter, it seems reasonable to consider whether the gonococcal propensity to shed outer membrane blebs or immune-stimulatory peptidoglycan fragments (176), genomic DNA (177, 178) and/or heptose phosphates (179, 180), or to directly engage with immune-regulatory surface receptors (110, 111) may contribute to this manipulation of the immune response, but the contribution of each remains to be experimentally tested.

Through these studies, a pattern is emerging to suggest that *N. gonorrhoeae* has developed a remarkable capacity to manipulate the immune response for its own benefit, suppressing adaptive Th1- and Th2-governed responses and concomitantly eliciting Th17-driven innate responses that it is able to resist (166). The gonococcus also avoids antigen recognition by extensive variation of both the expression and specificity of several major surface antigens, and it exploits certain aspects of innate immunity, including complement C3 receptors and CEACAM molecules to attach to and invade human cells (86, 94). Moreover, it resists destruction by innate defense factors such as antimicrobial peptides and the lytic action of complement, as well as intracellular killing by neutrophils (181, 182). A corollary of these findings is that if Th1- and/or Th2-driven adaptive immune responses can be induced against constitutively expressed common antigens, then it might be possible to overcome the limitations of innate defense mechanisms by adding antigen-specific factors such as antibodies. Recent findings support these notions, and suggest that the development of an effective vaccine may indeed be feasible.

RECENT VACCINATION EFFORTS

The induction of adaptive immune responses by administering microencapsulated IL-12 during gonococcal infection, discussed above, suggests that IL-12 functions as an adjuvant by turning the infection into a live vaccine. This finding led to the hypothesis that mice might be effectively immunized intravaginally with a vaccine consisting of gonococcal outer membrane vesicles (OMV), which retain most of the surface antigens of *N. gonorrhoeae*, plus microencapsulated IL-12 as an adjuvant (183). As predicted, this resulted in the generation of serum and genital anti-gonococcal antibodies, IFNγ production by CD4+ T cells, the establishment of immune memory that can be recalled by challenge infection 1–6 months later, and faster clearance of the challenge infection. Furthermore, the induced antibodies cross-reacted with diverse

strains of *N. gonorrhoeae*, and resistance to infectious challenge was shown against heterologous as well as homologous strains (183). This study provides a clear demonstration that immunity is achievable if a suitable immunogen is appropriately administered.

The product pipeline for gonococcal vaccines is currently in the "discovery phase," focused on the identification of vaccine targets and immune correlates of protection that they might confer (184). Progress in antigen discovery is ongoing, and has led to the identification of several conserved, stably expressed, promising vaccine targets [extensively reviewed in (181)]. Most of these antigens induce bactericidal antibodies against gonococci; some block target function, and some have shown *in vivo* efficacy in the female mouse model (32, 183, 185, 186). Candidate antigens involved in gonococcal physiology or metabolism include the transferrin receptor TbpA/B (187), which enables *N. gonorrhoeae* to access human transferrin-bound iron and is essential for urethral experimental infection of human male subjects (188), the methionine receptor MetQ (NG02139) (189, 190), nitrite reductase (AniA) (191), and phospholipase D (192). Other promising new candidates identified using a non-biased proteomics screen are involved in membrane biogenesis (BamA), LOS assembly (LptD), or translocation assembly (TamA) (190). Vaccine targets that mediate evasion of host innate defenses include MtrE, the outer membrane channel of the MtrCDE active efflux pump (193), the lysozyme inhibitor SliC (NGO1063) (190), and the Neisseria adhesion complex protein (ACP) (194). Two antigens that were identified by immunoproteomics as targets of antibodies induced by intravaginal immunization of mice with gonococcal OMV plus microencapsulated IL-12 are elongation factor (EF)-Tu and PotF3, a polyamine-binding protein (183). Both of these were also identified in proteomics profiling of cell envelopes and OMVs from *N. gonorrhoeae* strains (195). It is likely that additional targets might be identified by further immunoproteomics screening of sera and secretions taken from OMV-immunized animals.

Colonization factors are also attractive vaccine targets due to their potential to induce antibodies that block the initial establishment of infection. While pilin, the major subunit of the gonococcal pilus, is too antigenically variable to be effective, PilQ, which is the pilus secretin and thereby essential for pilus function, is an attractive target because antibodies against meningococcal PilQ are bactericidal (196). The gonococcus expresses several other outer membrane proteins that mediate adherence and/or invasion of host cells. Those for which antibodies have been shown to be bactericidal or to block interactions with host cells include OpcA (196), OmpA (197), PorB (185, 198), and the opacity (Opa) proteins (199–201). While enticing as a target because they are highly expressed on the bacterial surface and presumed to be essential for infection, as discussed above, PorB and Opa proteins are highly antigenically variable and the latter are also subject to phase variation. Moreover, while Opa expression is selected in the male urethra (96), it is selected against during menses in women (95), and by hormonally driven factors in experimentally infected female mice (125, 202). These findings illustrate that abundant and highly immunogenic surface antigens are not necessarily good vaccine targets.

As detailed above, the heptose-linked 2C7 epitope within the outer core of the gonococcal LOS is an enticing vaccine target. While it is phase-variable, there is selection for gonococci that express this epitope during human infections (31). Supporting its utility as a target, passive delivery of 2C7 monoclonal antibody is protective in mice (32). While in general polysaccharides tend to induce IgM responses with limited memory, a 2C7 peptide mimic has been developed which elicits IgG antibodies that are highly bactericidal and promote opsonophagocytic killing of gonococci, as well as *in vivo* protection against gonococcal challenge of mice (32). Interestingly, a hexamerized 2C7 monoclonal antibody that has enhanced C1q binding activity relative to the monomeric form showed increased protective activity against gonococcal infection in mice, and this was attributable to complement-mediated bactericidal activity (203).

Immune correlates and determinants of protection in the mouse model should begin to emerge as more antigens are tested for efficacy *in vivo*. Thus far, the 2C7 peptide mimic is the only vaccine for which there is strong *in vivo* evidence showing that bactericidal activity can contribute to protection against *N. gonorrhoeae* (32, 203). Antigens that showed protection against experimental murine infection in which T cell responses were examined or inferred from IgG1/IgG2a ratios include the 2C7 peptide mimic (32), gonococcal OMVs given vaginally with microencapsulated IL-12 (183), and recombinant, refolded porin (rrPorB) administered using a viral delivery system followed by rrPorB protein boosts (185). Whether serum bactericidal activity will also correlate with, or confer protection against *N. gonorrhoeae* as a result of immunization with different vaccine antigens remains to be seen. Meanwhile, broad immunologic parameters should be evaluated in future vaccine studies to help expand our understanding of these relationships.

While subunit vaccines may be effective against gonorrhea, either alone or in a "cocktail" of multiple antigens, OMV vaccines also represent an attractive approach as they contain many of the gonococcal surface antigens in their natural conformation. This approach is supported by epidemiological evidence suggesting that vaccination of humans with OMVs of the related species *N. meningitidis* might provide some protection against gonorrhea. This cross-sectional study in New Zealand examined gonorrhea rates in adolescents and adults aged 15–30 years who were given three doses of the OMV-based group B meningococcal vaccine MeNZB (204). Compared to cases of chlamydia as a control, a 31% lower rate of diagnosis with gonorrhea was found in vaccinated individuals during the follow-up period. Unfortunately, protection declined with time elapsed after immunization and was not observed in individuals who also tested positive for chlamydial infection. It is provocative that Bexsero, a recently approved serogroup B meningococcal vaccine that includes the same meningococcal OMV as used in MeNZB, does elicit antibodies that bind to *N. gonorrhoeae* (205), although serum from such individuals does not induced complement-mediated bacteriolysis of gonococci (206). Further work is required to determine whether MeNZB immunization played a causal role in the decline of gonorrhea since, for

TABLE 2 | Pre-clinical models and pathway for gonococcal vaccine development.

Model/species	Objectives/advantages	Remarks/limitations
Mouse	Demonstrate contribution of non-host-restricted vaccine targets to infection or disease	Numerous defined antigens proposed based on *in vitro* studies, genome-mining, and reverse-vaccinology, but few have been evaluated so far
	Evaluate alternative or engineered immunogens	Allows prioritization of immunogens
	Evaluate alternative adjuvants	Different adjuvants may be appropriate for different immunogens
	Evaluate dose (antigen, adjuvant) and immunization routes and schedule	Will need to be re-evaluated in humans
	Determine conventional mechanisms and correlates of protection	May not fully reflect mechanisms in humans
	Identify potentially harmful antigens	Rodent toxicology may not be fully applicable to humans
Transgenic mice expressing human receptors	As above but overcomes limitations imposed by murine receptors and proteins not recognized by *N. gonorrhoeae*	Allows interaction between vaccine targets and their human receptors to be considered
Humanized immune system mice (SCID mice engrafted with human hematopoietic stem cells)	Evaluate potential responses of engrafted human immune system to *N. gonorrhoeae in vivo*	Murine genital tract imposes limits on gonococcal infection; unclear how effectively human cells are recruited to mouse tissues
Non-human primates (monkeys)	Determine cell-mediated and antibody responses	Monkeys not yet shown to be susceptible to genital gonococcal infection, thus protection not testable
	Evaluate toxicity and reactogenicity, especially in female genital tract	
In vitro cell and tissue culture (human)	Establish function of gonococcal virulence factors, and potential protective mechanisms based on responses identified in animals	Primary cells preferable to transformed cell lines
Humans (experimentally infected, male only)	Determine immune responses in early stages of infection	Findings may not be applicable to women
Humans (naturally infected, male and female)	Determine humoral and cellular (cytokine) responses in all stages of natural infection	Need to be re-evaluated in light of current knowledge and techniques; long-term studies not ethically permissible

example, this could also have been attributable to continued nasal carriage of the epidemic meningococcal strain in the study population (207), or whether the apparent protection is actually attributable to cross-reactive antibody or T cell responses induced by the vaccine.

SUMMARY AND FUTURE DIRECTIONS

Observations obtained from any experimental model, ranging from human cells or cell lines to animal infections, must be taken in context, understanding both the strengths and weaknesses of each model, and appreciating that natural infections may have different outcomes because models cannot faithfully reproduce all aspects of the relationship between a pathogen and its host. This is particularly true when their evolutionary paths overlap as much as those of *N. gonorrhoeae* and humans. Indeed, the strict specificity of *N. gonorrhoeae* for life in humans necessitates that we use every available tool to understand the molecular, metabolic, and immunologic interactions that determine the outcome of infection. As discussed throughout this review, neisserial researchers have been extraordinarily resourceful in this regard, developing infection models based upon genetically tractable cell lines; primary human epithelial cells and leukocytes; fallopian tube organ culture and cervical tissue explants; wild type, knockout and "humanized" transgenic mouse lines; and primate and human male urethral challenge models to study different aspects of infection (summarized in **Table 2**). When considered together, the findings from these different approaches allow a picture of the gonococcal lifestyle to begin to emerge, both remarkable in its elegance and devastating in its effectiveness as a human parasite.

Future studies must aim to further integrate these models, using each to develop new hypotheses and to validate findings observed in other systems. As this happens, each can become more sophisticated and the implications of any observations will be more effectively understood in the context of gonococcal infection and immunity. For example, ongoing efforts to "humanize" the mouse rely on cell line and primary cell-based efforts to understand gonococcal association with human cellular receptors (94), as well as blood-based studies to understand its ability to avoid serum bactericidal activity (208). Similarly, ongoing efforts to understand and then exploit gonococcal genetics have allowed *in vitro* microbiological approaches to reveal metabolic and drug resistance mechanisms that have led to studies in the established experimental human infection models (188, 209).

While not without their own pitfalls, murine infection models represent a bridge between *in vitro* cell-based studies and *in vivo* human infection. As discussed in this review,

recent mouse-based studies provide a plausible explanation for why individuals with gonorrhea do not become immune to subsequent infection. This model is intellectually satisfying because it affords a hypothesis that explains how *N. gonorrhoeae* can repeatedly infect individuals and thereby persist in the population, and it also provides insight as to what types of immune response might be effective in combatting the pathogen. Future mouse-based studies should reveal whether this outcome reflects the normal response of the female genital tract to bacteria, or whether it represents instead a unique characteristic of *N. gonorrhoeae*, providing fundamental insight regarding infection, immunity, and immuno-pathogenesis. Taken together, the findings obtained from all experimental systems, including *in vitro* cell- and tissue-based systems, animal models, and human studies afford a novel paradigm within which to formulate and refine new hypotheses for testing, thereby providing new insights into this complex and vexing infection.

The ultimate goal of these efforts is to develop new interventions to stop the spread of *N. gonorrhoeae*, an aim that is particularly enticing considering that the restricted host niche might make it possible for this pathogen to be eradicated once a vaccine is in hand (210, 211). While we have passed through a period in which gonorrhea was often considered as a low priority afterthought, the recent rapid increase in rates of infection and the emergence of multidrug-resistant "superbug" strains of *N. gonorrhoeae* has re-awoken the public's appreciation of this emerging threat, and re-invigorated public health and industrial interest in developing a gonococcal vaccine. This attention has come at an opportune time, since recent work has offered new insight into what types of response can confer protection, revealed new targets for vaccine development, and provided ever-more sophisticated models in which to test their efficacy. Hopefully, this momentum will rapidly bring us to a gonococcal vaccine.

AUTHOR'S NOTE

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Conflict of Interest: MR serves as a paid consultant for TherapyX, Inc., which is developing sustained release microparticulate adjuvants for use in inflammatory disease therapy and gonococcal vaccine development. SG-O is co-founder of Engineered Antigens Inc., which is focused on protein structure-based design of vaccine immunogens targeting pathogens including *N. gonorrhoeae*.

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REVIEW
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Exploitation of *Neisseria meningitidis* Group B OMV Vaccines Against *N. gonorrhoeae* to Inform the Development and Deployment of Effective Gonorrhea Vaccines

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Have potential clues to an effective gonorrhea vaccine been lurking in international disease surveillance data for decades? While no clinically effective vaccines against gonorrhea have been developed we present direct and indirect evidence that a vaccine is not only possible, but may already exist. Experience from Cuba, New Zealand, and Canada suggest that vaccines containing Group B *Neisseria meningitides* outer membrane vesicles (OMV) developed to control type-specific meningococcal disease may also prevent a significant proportion of gonorrhea. The mechanisms for this phenomenon have not yet been elucidated but we present some strategies for unraveling potential cross protective antigens and effector immune responses by exploiting stored sera from clinical trials and individuals primed with a meningococcal group B OMV vaccine (MeNZB). Elucidating these will contribute to the ongoing development of high efficacy vaccine options for gonorrhea. While the vaccine used in New Zealand, where the strongest empirical evidence has been gathered, is no longer available, the OMV has been included in the multi component recombinant meningococcal vaccine 4CMenB (Bexsero) which is now licensed and used in numerous countries. Several lines of evidence suggest it has the potential to affect gonorrhea prevalence. A vaccine to control gonorrhea does not need to be perfect and modeling supports that even a moderately efficacious vaccine could make a significant impact in disease prevalence. How might we use an off the shelf vaccine to reduce the burden of gonorrhea? What are some of the potential societal barriers in a world where vaccine hesitancy is growing? We summarize the evidence and consider some of the remaining questions.

Keywords: gonorrhea, OMV vaccine, MeNZB, *Neisseria meningitides*, *Neisseria gonorrhoeae*

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LESSONS FROM *N. MENINGITIDIS*

Unlike *Neisseria meningitidis* serogroups A, C, W, and Y, for which effective polysaccharide-protein conjugate vaccines have been developed, serogroup B has required alternative strategies. This is because of the poor immunogenicity of the group B capsular polysaccharide and its likely homology to fetal neural tissue (1). The original solution to this problem was the development of group B

strain specific vaccines based on the outer membrane vesicle (OMV). These vaccines are based on the immunodominant protein Porin A (PorA), which is highly variable across strains, therefore their use has traditionally been considered restricted to situations where disease is dominated by a single PorA strain (2, 3). Reports on the duration of protection afforded by OMV vaccines against meningococcal disease vary according to age at vaccination and the target population, but serum bactericidal activity (SBA), which is the correlate of protection, has typically diminished among a significant proportion of vaccinees by 2 years, due to the waning of serum antibody (4).

While devastating, meningococcal disease is rare and meningococcal vaccines rely on immunogenicity data as a proxy for likely efficacy (5). This is because a randomized efficacy trial powered to detect meningococcal disease cases as a primary outcome would need in the order of 100,000 participants (6–8), rendering this approach unaffordable and impractical. Fortunately the presence of SBA provides a correlate of protection that can be used to estimate efficacy (5, 9, 10).

Estimating both the efficacy and effectiveness of meningococcal vaccines directly is hindered by the low case numbers. Both efficacy and effectiveness are generally estimated by calculating the risk of disease among vaccinated and unvaccinated persons and determining the percentage reduction in risk among each group relative to each other. Because of the low case numbers, and in the case of polysaccharide-protein-based, the significant impact on carriage (11–13) associated with meningococcal vaccines the estimates have wide confidence intervals (14).

Ultimately the public health value of these vaccines is revealed by real world experience and ecological observations on overall incidence and prevalence of disease which indirectly supports the vaccine impact. Where meningococcal vaccines are concerned estimates of 70 or 80 percent efficacy may translate to much higher effectiveness and near elimination of disease (12, 15–17).

Because the immunogenicity, efficacy, and effectiveness of OMV vaccines has generally been considered limited in terms of strain and type coverage compared with conjugate vaccines, and they have not been as widely used with less data published compared with their purer conjugated relations. While immunogenicity of OMV vaccines generally predicts efficacy (particularly for younger age groups) it may be less predictive of effectiveness (5). Less explored too are the minor components present in the OMV and their potential role in inducing not only protection against homologous PorA types but heterologous protection against a range of *Neisseria*. The traditional IgG activated complement mechanism that has perhaps driven and dominated the meningococcal vaccine field may miss a cocktail of novel antigens with powerful adjuvant effects (18).

POTENTIAL IMPACT OF A GONOCOCCAL VACCINE

An effective vaccine does not have to be a highly efficacious vaccine. When the basic reproduction number of an infection is low, and a vaccine affects transmission, then disease control can be achieved with a vaccine that has moderate efficacy. While vaccines are given to individuals to protect them against disease, many vaccines also reduce transmission, thereby protecting the broader community (19). Consideration of a vaccine's effect on carriage and transmission is a vital component of immunization programme planning.

The basic reproduction number (R_0) describes the maximal potential for spread of an infection within a population. It depends on the contact rate, the duration of the infectious period and the probability that the contact between an infectious person and a susceptible person leads to an infection (19). For the most infectious diseases (measles and pertussis) a single infectious individual entering a community of non-immune individuals can infect a further (R_0) 12–18 and 5–17 people, respectively (20). Effective vaccines against these diseases need to be highly efficacious and affect transmission in order to successfully control or eliminate disease. They also need to provide sustained immunity, or be given as regular boosters, and be administered to 92–94% of the population (19).

In contrast, mumps has an R_0 of around 4–7 and influenza 1.4–4. The proportion of the community that needs to be immune to prevent transmission of these diseases is 75–85 and 30–75%, respectively (20). Estimates of R_0 for gonorrhea are in the order of 1.18–3.6 depending on the method used. The lower estimates rely on the assumption of acquired immunity after infection, the higher estimates do not assume complete acquired immunity and are therefore likely to be more accurate (21).

Using an individual-based, epidemiological simulation model the potential impact of a hypothetical gonorrhea vaccine was modeled and the prevalence of gonorrhea in a heterosexual population estimated using various assumptions of efficacy and duration of protection. The individual-based approach applies the dynamics of the *probability* that an individual is in a certain state (susceptible, infected, recovered) as opposed to a static network where an individual is in one of the states (22). In this study it was assumed that there was no immunity following resolution of natural infection. The modeling demonstrated that a vaccine of moderate efficacy could have a significant impact on the prevalence of gonorrhea if strategically implemented (23). While encouraging it does, of course, depend on the availability of a vaccine.

FROM ECOLOGICAL DATA TO EVIDENCE

The epidemiological evidence from Cuba, Brazil, and New Zealand demonstrates that *N. meningitidis* OMV vaccines are possibly able to provide some broader protection against meningococcal disease (17, 24), particularly in older children and adults (25). These observations led to the hypothesis that they may affect a more distantly related bacterium. Examination of surveillance data clearly show a marked decline in the incidence of gonorrhea in Cuba following implementation of the VA-MENGOCBC (**Figure 1**). The pattern of decline in incidence for gonorrhea contrasts with syphilis and genital warts for which incidences have remained the same (8). This phenomenon was also observed in NZ, where a decline in reported gonorrhea cases

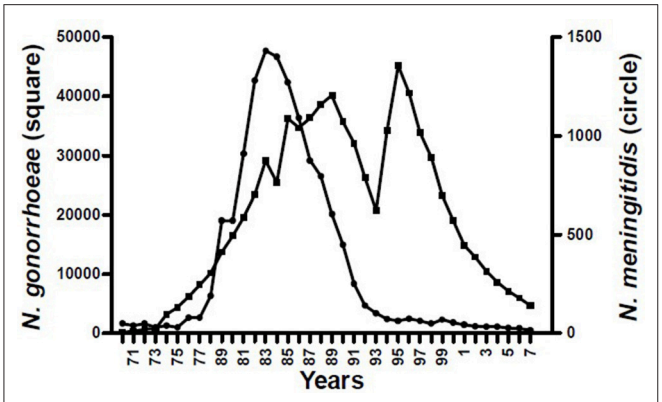

FIGURE 1 | Morbidity of *Neisseria* pathogenic species in Cuba 1970–2007 (8).

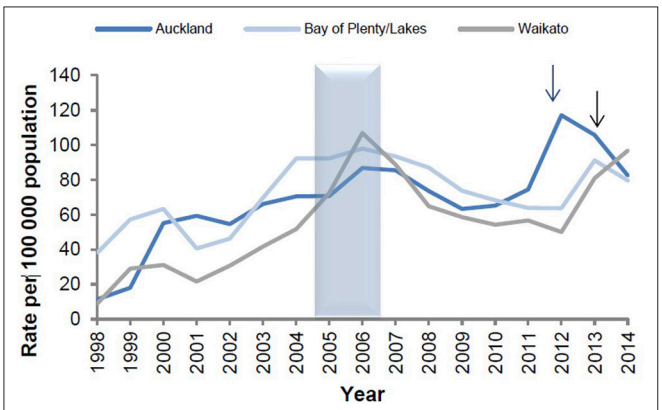

FIGURE 2 | Reported gonorrhea rates in New Zealand 1998–2014, selected sentinel regions (26). Arrows denote shifts from culture testing to NAAT testing.

during and shortly after use of the tailor made meningococcal Group B OMV vaccine MeNZB is evident (**Figure 2**). Like Cuba, no other sexually transmitted infections (STIs) described in the national surveillance reports declined during that period (26). While purely ecological, these observations suggested that *N. meningitidis* OMV vaccines might offer cross protection against gonorrhea.

This hypothesis was first tested in New Zealand where both the MeNZB exposure and gonorrhea outcome data could be linked in a retrospective case-control study. The demographic details and vaccine status of 14,730 deidentified sexual health clinic patients aged 15–30 years, who had been eligible to receive the MeNZB vaccine, were determined via the linkage to the country's National Immunization Register and database of demographic information. The outcomes of interest were laboratory confirmed gonorrhea and, as a control, chlamydia. The odds of disease outcomes in vaccinated and unvaccinated patients were compared. Individuals who had received the MeNZB vaccine were significantly less likely to be gonorrhea cases than chlamydia controls, with an adjusted OR 0.69 (95% CI 0.61–0.79%); $p < 0{\cdot}0001$. This translated to a vaccine effectiveness estimate of 31% (95% CI 21–39) (27).

Following on from the case-control study a national cohort study of gonorrhea hospitalizations was conducted. The eligible cohort consisted of 1,143,897 individuals born 1984–1999 residing in New Zealand during 2004–2008 and therefore eligible to receive the MeNZB vaccine during that time. In this study administrative datasets of demographics, customs, hospitalization, education, income tax, and immunization, were linked using a national data collection called the National Integrated Data Infrastructure. The primary outcome was hospitalization with a primary diagnosis of gonorrhea. Using Cox's proportional hazards models with a Firth correction for rare outcomes, estimates of hazard ratios were generated. Vaccine effectiveness estimates were calculated as 1-Hazard Ratio expressed as percent. After adjustment for gender, ethnicity and deprivation, MeNZB vaccine effectiveness against gonorrhea associated hospitalization was estimated to be 24% (95% CI 1–42%) for the whole eligible cohort and 47% (95% CI 18–66) for those vaccinated in adolescence and therefore most likely to be at risk for gonorrhea during the follow-up period (28). While limited by small numbers the findings supported the earlier case-control study.

The MeNZB vaccine was developed to control a meningococcal group B epidemic and it is no longer available. However, the same OMV used in the MeNZB vaccine is included in the new generation meningococcal group B 4CMenB (Bexsero) vaccine. Bexsero also includes three recombinant proteins that are conserved across *N. meningitides* [Neisserial Heparin Binding Antigen (NHBA), factor H binding protein (fHbp), and Neisseria Adhesion A (NadA)], two of which are shared variably with Neisseria *gonorrhoeae (N. gonorrhoeae)* (29). NHBA and fHbp, along with accessory fusion proteins GNA1030 and GNA2091 in the formulation, are capable of inducing immune responses against the gonococcus (30, 31). If additional immunogenicity and cross protection is afforded by this new generation vaccine, on account of the additional antigens and synergistic combination, then the potential for effectiveness is likely to be higher than that demonstrated by the MeNZB vaccine.

A mass vaccination campaign in Quebec, Canada, using Bexsero, provided an opportunity to observe a potential vaccine effect on gonorrhea. In 2014 Bexsero was administered to individuals aged 6-months to 20-years of age. Gonorrhea and chlamydia notifications to public health authorities during the pre-vaccination period and post-vaccination period (2006–June 2017) were analyzed and the impact of the campaign estimated by a Poisson regression model. Vaccine coverage was 82% in the target group. There were 231 gonorrhea cases reported among persons 14 years and older (IR: 8.4/100,000 person-years) in the region of the mass campaign during the study period. A decrease in the number of cases and risk among individuals 14–20 years was observed during the post-vaccination period. In contrast, it increased in those 21 years and older. As observed in both New Zealand and Cuba, chlamydia infections increased among both the vaccinated and unvaccinated age groups. The estimate of vaccination impact was a risk reduction in gonorrhea of 59% (95% CI: −22 to 84%; $P = 0.1$) (32). While the confidence intervals

are wide and include "0" this is likely due to the low study power.

Together the NZ, Cuban, and Canadian data suggest that we likely already have a vaccine to hand, that if used strategically, could impact on the prevalence of gonorrhea. But what are the mechanisms?

STRATEGIES TO DEFINE THE MECHANISMS OF CROSS-PROTECTION

The intriguing observation that vaccination with meningococcal B OMVs confers a degree of protection to gonorrhea raises some interesting questions about possible mechanisms of action. Historically there have been some major confounders for development of a gonococcal vaccine—earlier trials proved unsuccessful, there are no defined correlates of protective immunity and the optimum route of delivery is unknown. The MeNZB data suggest it may be possible to elicit protective immunity to gonococci with a parenteral vaccine and there is now an opportunity to examine resources generated both from the clinical trials of this vaccine and New Zealand's large MeNZB primed cohort for clues as to how this vaccine confers this effect.

The lack of known correlates of protective immunity is a major stumbling block to identifying vaccine candidate antigens or novel approaches for developing a gonococcal vaccine. Population data show that prior exposure does not protect individuals from re-infection (33) and human challenge studies have demonstrated that recently infected individuals remain susceptible to re-infection with the challenge strain (34). Longitudinal studies of individuals regularly infected with gonorrhea have proved invaluable in demonstrating how host antibody mediated responses to selected antigens have been used to subvert the immune response. Gonococci can be typed into serovars on the basis of expression of variants of a major outer membrane protein, porin (PorB). Both *porB* typing alone or in combination with additional molecular analyses have shown that *por* mutates in response to immunological pressure, suggesting that the effective immunity to gonococci may develop, but is confounded via a shifting of phenotype by the bacteria (35, 36). Development of an elevated antibody response to another major outer membrane protein, reduction modifiable protein (Rmp)/protein III, is associated with enhanced susceptibility to infection (37). *In vitro* studies have shown that IgG antibodies to Rmp prevent other potentially protective antibodies from initiating bactericidal activity to gonococci (38). However, individuals with high occupational exposure to gonococci do eventually develop serovar-specific immunity (35), suggesting a vaccine may ultimately be feasible (39). Experimental, self-limiting infection of human subjects with gonococci results in development of modest local and systemic specific antibody responses, with a suggestion that serum antibody responses to lipo-oligosaccharides (LOS) may confer a degree of protection from re-infection (34).

Several prospective gonococcal vaccines have been tested in humans without success. To date these attempts have included parenteral administration of non-adjuvanted material including partially lysed whole bacteria (40), pilin (41), and protein I/PorB, a major porin/outer membrane protein [reviewed in (42)]. One of the most promising pre-clinical vaccine candidates identified to date is a highly conserved oligosaccharide epitope (2C7) common to most gonococcal isolates—targeting this epitope elicits sialylation-independent bactericidal activity *in vitro* and leads to enhanced clearance of infection in mice (43, 44). Pre-clinical evaluations suggest that gonococcal OMVs are also a promising vaccine candidate (45–47) but they have not been tested in humans. In contrast serogroup B *N. meningitidis* was the first organism to be screened for meningococcal vaccine candidate antigens using a sophisticated genomic-led approach termed "reverse vaccinology," which led to the selection of the highly conserved vaccine candidate antigens incorporated into Bexsero (48). Importantly though for full efficacy and enhancement of coverage to a broader range of subserotypes and better immunogenicity in younger age groups, this vaccine still requires the addition of OMV from MeNZB, with porin A considered to be a particularly important constituent (49, 50).

The OMVs are comprised of a complex mix of periplasmic, cytoplasmic, and outer membrane proteins (51). The dominant components include outer membrane proteins such as porin A, porin B, Rmp, NspA, and the OpcA invasin which are incorporated into vesicles in conjunction with lipopolysaccharide [summarized in (52)]. An immuno-proteomics analysis of the related Cuban meningococcal OMV vaccine suggests that the antibody response is primarily targeted to these major antigens (53). Population genomics has demonstrated the close genetic relationship between *N. meningitidis* and *N. gonorrhoeae* (50) suggesting OMVs are likely to be the source of numerous conserved vaccine candidates, both protein and glycolipid. Screening of gonococcal OMVs from multiple strains with human anti-meningococcal sera would be an effective approach to identify cross-reactive, conserved "human relevant" antigens. Although this approach is likely to reveal multiple cross-reactive antigens, some of which are unlikely to be suitable vaccine candidates, selection of novel candidate antigens can be informed and complemented by the rapid expansion of knowledge of gonococcal biology. For example, conserved novel antigens of importance for survival of the gonococcus have recently been identified by quantitative proteomics, which has successfully been applied to cell envelope and OMVs from multiple strains of *N. gonorrhoeae* (54).

As *N. gonorrhoeae* is an exclusive human pathogen the relevance of animal models, particularly non-primate models, is contentious. Female mice can be transiently infected with *N. gonorrhoeae* if they are pre-treated with estradiol and inoculated during the pro-estrous phase (55), but lack important features such as carcinoembryogenic antigen-related cell adhesion molecules (CEACAM; CD66) on neutrophils and epithelial cells, which are typically targeted by the gonococcal opacity associated proteins (Opas) (56, 57). Gonorrhea infection in mice does recapitulate several known features of human infection, eliciting a polarized neutrophilic Th17 driven response and production of pro-inflammatory cytokines (58–60). Conversely, accelerated clearance of the infection is linked to a Th-1 driven immune response in mice, particularly the production

of interferon-γ, in conjunction with the development of both systemic and local IgG and A (45, 61–63). Of note, vaginal vaccination with gonococcal OMVs delivered with encapsulated IL-12 can elicit enduring protection to multiple distinct gonococcal isolates (45) and intranasal administration of gonococcal OMVs can expedite resolution of infection (46), however it is not yet known whether parenteral vaccination with either gonococcal or meningococcal OMVs can elicit a comparable effect.

Although gonococci can infect other mucosal sites, the most important site of entry is the genital mucosa. Unlike other mucosal locations, the genitals lack inductive sites for local antibody production and IgG (produced locally and systemically), not IgA, is considered to be the most important class of antibody (64). It has been traditionally accepted that the optimal way of developing vaccine-elicited immunity to mucosal pathogens is to immunize by the mucosal route, preferably one which mimics the natural route of infection (65). Accordingly there are several examples of highly effective vaccines—most notably the live oral vaccine for poliomyelitis—which are administered orally, although further refinements in this arena have been stymied by the lack of safe, effective mucosal adjuvants. More recently it has been proposed that parenteral vaccines may be an equally feasible means of stimulating strong and appropriate protective mucosal responses (66). Of particular relevance to gonorrhea, the Human Papillomavirus (HPV) vaccine is comprised of an adjuvanted preparation of virus-like particles given intramuscularly, which results in the development of enduring antibody responses in both systemic (serum) and local (cervicovaginal) sites, with a strong correlation between IgG levels in the two locations, supporting the possibility of serum transudation or exudation into the genital mucosa (67). Evidence from gonococcal vaccine studies in mice also indicate that antibody responses—including systemic IgG responses—are associated with protective immunity to gonococci, suggesting serum antibody responses can reasonably be used as a correlate of protection (45).

But how does MeNZB, which is reported to confer a relatively short-lived period of protective immunity in infants and toddlers (4, 68), confer cross-protective immunity to gonococci for some years after vaccination? An elevated SBA is the benchmark for verifying sustained protective immunity to group B meningococci, but there may well be other aspects of the vaccine initiated immune responses that are equally or more important for development of immunity to gonococci. Specific mucosal immune responses are likely to be necessary for limiting the development—and ideally transmission—of symptomatic gonorrhea infections. While the systemic antibody responses elicited by MeNZB have been thoroughly examined, there have been no long term studies on the development of the mucosal antibodies in response to vaccination. An assessment of adults shortly after vaccination showed either no change or very modest increases in salivary antibody responses in response to parenteral vaccination with the Norwegian MenBVac (69) or MeNZB (70). Conversely, anti-meningococcal salivary IgA responses were reported to increase with age and/or meningococcal carriage (71), suggesting that an examination of the long-term effects of vaccination in childhood may yield useful information on whether nasopharyngeal acquisition and/or carriage of commensal *Neisseria* can boost mucosal antibody responses. Given the issue of rising rates of oral gonorrhea and evidence of oral-genital transmission of infection (41), induction of strong mucosal immune responses in both sites is likely to be important.

Analysis of specific cellular responses in the tonsils suggest that parenteral vaccination of adults with MeNZB results in re-programming of the mucosal immune response to meningococci in the nasopharynx (70). OMVs contain a complex package of virulence factors, TLR agonists, and other secreted or membrane associated components that interact and modulate host immunity (72). Detergent extracted MeNZB OMVs are reported to consistently contain ∼100 distinct proteins predominantly from the outer membrane compartment (73) and although delivered with an adjuvant, they are also intrinsically immunogenic. Perhaps OMVs from mucosal pathogens such as *Neisseria* have the capacity to stimulate homing and development of immune effector cells at mucosal sites after parenteral immunization? This may be important for eliciting strong mucosal immune responses (66) as has recently been reported for the detoxified form of heat-labile toxin (dmLT) (74) and linked to properties of the adjuvant itself, rather than the route of administration. This would be an interesting concept to explore further in mice vaccinated parenterally or mucosally with OMVs; or translated to humans through application of emerging technologies in the immunogenomics and systems biology arena (75) with the potential to provide vital information to support the utility of this class of vaccines.

Human mucosa, particularly the oropharynx, is frequently host to several different species of *Neisseria*. Genetic analyses of both pathogenic and non-pathogenic *Neisseria* show they are closely related, with evolutionary studies suggesting frequent exchange of genetic material including virulence genes (76). Notably, early exposure to commensals such as *N. lactamica* may not only confer some degree of protection from *N. meningitidis*, but could be an approach for identifying novel vaccine candidate antigens (77). Carriage of non-pathogenic *Neisseria* may also enhance the development of immunity to *Neisseria* OMV vaccines (78). *N. meningitidis* and *N. gonorrhoeae* are most closely related and primarily separated from the remaining members of the genus by the presence of additional virulence genes (76). The impact of vaccination with meningococcal OMVs, which contain multiple highly conserved antigens common to many *Neisseria* species, on nasopharyngeal colonization has not yet been determined. A genetic analysis of the additional antigens in Bexsero suggest that this vaccine could impact non-target *Neisseria* species as both NHBA and the additional fusion proteins GNA1030 and GNA2091 contained in Bexsero are highly conserved across both pathogenic and commensal *Neisseria*, whereas *N. gonorrhoeae* does not express NadA and this bacterium contains only one of three possible variants of fHbp (79, 80). It has been suggested that NHBA could form the basis of a putative gonococcal vaccine, but would likely require

augmentation with additional conserved proteins to enhance the effectiveness of such a vaccine (80). MeNZB anti-sera is highly likely to be a useful source for identifying additional highly conserved gonococcal immunogens. The utility of this approach has been demonstrated in rabbits, which develop cross-reactive antibodies to several strains of gonococci by ELISA and western blot in response to vaccination with MeNZB/Bexsero OMVs (31). Human MeNZB anti-sera will almost certainly show a similar level of cross-reactivity, with the potential complication of reactivity to antigens common to commensal *Neisseria* species.

New Zealand currently has a large cohort of individuals at or approaching the age of prime interest for studying the long-term impact of the MeNZB vaccine. Notably there is an opportunity to examine recall responses ∼10 years post-priming in a population of adolescents and/or young adults, which is a good match for the likely timing of administration of a gonococcal vaccine and corresponds with the age groups at greatest risk of contracting gonorrhea. The impact of boosting these individuals with Bexsero (as the source of MeNZB OMVs) on both local and systemic antibody responses as well as the development of cellular immunity has the potential to provide valuable data on the possible targets or mechanisms of cross-reactive immunity. A large collection of sera also remains from the original MeNZB clinical trials, which were commenced in adults (81), followed by pre-teens (82), toddlers then infants (83) with serum samples obtained prior to and at regular intervals during vaccination. Untouched duplicate samples were retained after the completion of these trials and can be accessed for investigation of anti-gonococcal responses. Meningococcal SBA titres were quantified as part of the MeNZB vaccine development programme, which offers a unique opportunity to not only determine whether MeNZB vaccination elicited a bactericidal antibody response to gonococci in humans, but also to ascertain whether this correlates with elevated anti-meningococcal SBA titres.

A key question remains as to which approaches would be the most appropriate for examining possible cross-reactive immune responses to gonococci in these vaccinated individuals. Induction of functional antibody responses such as complement-mediated bacterial killing, inhibition of binding to reproductive tract epithelial cells and stimulation of opsonophagocytosis are generally considered to be suitable starting points. The development of an increased serum bactericidal antibody (SBA) response is the standard for determining the protective efficacy of vaccines to meningococci (84–86) whilst the presence of bactericidal antibodies to gonococci are frequently used as a likely surrogate of protective immunity in pre-clinical studies (47, 87, 88). Cross-reactive bactericidal antibodies are, unsurprisingly, likely to be directed to gonococcal lipopolysaccharides and surface proteins (89). Therefore, analyzing SBA responses is a valid starting point, although it will be important to incorporate multiple gonococcal strains to confirm broad, cross-protective bactericidal activity to gonococci. A limitation of the SBA is that it requires use of bacteria that are resistant to complement-mediated killing by normal serum, whereas serum sensitivity is reported to be common in gonococci after *in vitro* culture (90). Serum resistance can be induced by the use of additives to sialylate LOS (91, 92) and screening of resistant phenotypes is more likely to represent the low susceptibility to serum-mediated killing seen *in vivo*. Enhanced opsonophagocytosis is considered to be another likely correlate of protective immunity to gonococci and this can readily be inferred by detection of C3b deposition on the surface of the gonococci using flow cytometry (93), as a pre-cursor to MAC-mediated lysis, and corroborated by determining whether opsonophagocytic uptake of gonococci by a neutrophil-like cell line, such as retinoic acid differentiated HL-60 cells (94), is enhanced in the presence of immune sera.

A critical step in the pathogenesis of *N. gonorrhoeae* is adherence to target epithelial cells where initial adherence is predominantly mediated by pili, followed by tight attachment to CEACAM via expression of OpA. The interactions of gonococci with human reproductive tract cell lines (95, 96) and primary cultures (97) have been described and these model systems can be used to establish whether introduction of sera from MeNZB vaccinated individuals inhibits adherence or invasion of gonococci. These sera can also be used to determine whether there is a quantifiable increase in antibodies to gonococcal OMVs or cell surface exposed antigens by ELISA or development of cross-reactive antibodies to antigens conserved across multiple isolates of *N. gonorrheae* using similar immunoproteomics approaches to those applied to *N. meningitidis* (53, 98, 99). Both approaches can be used to identify or validate potential gonococcal vaccine candidates, in conjunction with pre-clinical testing in *in vivo* models.

There have been few studies on the development of cellular immune responses to MeNZB (70) or other meningococcal OMV vaccines in adults (100, 101). None have examined early immune kinetics in naïve individuals, the development of recall responses, or induction of responses to gonococcal OMVs. The type of cellular response required to prevent gonococcal infection in humans is unknown, but murine studies link development of protective immunity with Th-1 immune responses (45). An assessment of Th profiles in response to stimulation with gonococcal antigens could ascertain whether MeNZB vaccination causes a similar skewing in humans.

Mining resources from New Zealand's MeNZB vaccination programme may be fruitful for identifying potential correlates of immunity in human subjects. Ultimately a prospective clinical trial or high quality observational study with a large cohort of high risk individuals will be necessary to acquire a complete and accurate picture of how well a vaccine containing OMVs (or other potential antigens) will perform in reducing rates of gonorrhea.

SOCIETAL AND POLICY ISSUES IN GONOCOCCAL VACCINE DEPLOYMENT

Modeling has suggested that a vaccine with moderate protection might have a significant effect on the burden of gonorrhea (23). Considering Bexsero as a candidate intervention, how would we best use it?

While we do not yet know the mechanism of protection induced by these OMV vaccines that has resulted in some resistance to gonorrhea we might assume that it is not long lasting based on experience with protection against meningococcal disease as well as the waning observed in the New Zealand case-control study (27). There are still questions to be answered about the effectiveness of Bexsero. It does appear broadly protective against meningococcal disease, including against the hypervirulent Group W strain, which is increasing in prevalence in some countries (102, 103). However, to what extent this affects carriage in adolescents, and duration of protection at the population level remain to be demonstrated (103, 104). Therefore, in order to optimize protection against gonorrhea such a vaccine would need to be delivered prior to sexual debut and use of boosters possibly maintained throughout the risk period. Early adolescence also happens to coincide with a high risk period for meningococcal disease. Administering a dose of Bexsero during early adolescence in a population previously primed in infancy, or two doses in a previously naive population, might be a pragmatic strategy to reduce gonorrhea whilst at the same time improving the community immunity to meningococcal disease. If protection was demonstrated to wane during the risk period a further dose could be considered to maintain protection.

Even the most efficacious vaccine cannot prevent disease if it is not used. Funding and policy aside perhaps the greatest challenge facing immunization programmes today is the growing presence of vaccine hesitancy (105, 106). The dream of global measles and rubella elimination is unraveling as a tide of trolls, bots, and organized opposition, facilitated by social media, plays havoc with trust and confidence (107–109). Vaccine coverage rates have dived in many countries and measles resurgence is occurring among populations that had previously achieved elimination status, such as the America's and some European countries (110). How might a gonorrhea vaccine fair in this environment of growing public resistance to vaccines?

If we consider the societal factors that might be relevant to a vaccine against a disease that is largely sexually transmitted then we need look no further than the experience with the human papillomavirus (HPV) vaccines. The focus of the original marketing of HPV vaccines was prevention of cervical cancer as opposed to prevention of a sexually transmitted infection (111, 112) however, skepticism about the vaccine effectiveness and safety arose quickly from a variety of quarters (113, 114). Even with the focus on cancer there has been ongoing public outrage fed by organized lobby groups since 2007, when the first vaccine was licensed (115). After the introduction of HPV vaccine there was a shift toward a more conservative backing for the anti-vaccine movement. Presumably this was because despite the vaccine being marketed as an anti-cancer vaccine the fact that the virus is primarily sexually transmitted invoked discomfort among those with conservative and religious views about sex (116). Consequently whilst some countries have delivered to over 70–80% of the target population (117) others have fared less well with some countries either failing to implement a programme, or experiencing interruption to programs due to widespread movements aimed at discrediting the vaccine (118). Some countries have had their previously high coverage eroded to below 30% (119).

Given the multiple challenges in marketing and delivering a vaccine against a sexually transmitted infection across diverse cultures there may be an argument for desexualizing it and packaging it as a vaccine against *Neisseria*. However, efforts to desexualize vaccines can backfire. Withholding information that is seen to be less agreeable to the public can result in accusations of paternalism (116). This will likely result in the erosion of trust. How public health officials communicate the facts about a gonorrhea vaccine across multi-cultural societies will likely have an impact on acceptance.

Some societies have chosen to place HPV vaccine at the 9-year age mark, other societies have elected to place it at the 12–13 year mark. While the 9-year mark might desexualize the vaccine, the luxury of this choice is unlikely to be an option for a gonorrhea vaccine. The HPV vaccine, like the Hepatitis B vaccine, has a long duration of protection (120). It is unlikely that a gonorrhea vaccine based on current options will provide long-term protection, therefore placement in a national immunization programme will need to be at the age that provides highest immunity just before sexual debut.

While research continues into gonorrhea vaccine antigen discovery there is also a need for further data on the two OMV-containing *N. meningitidis* vaccines currently available (Bexsero and VA-MENGOCBC) that might have some utility if deployed into a sexually active population. For example, further knowledge about the effect of age and immunological experience on the vaccine response, along with boosting responses in older children, and adults who have been primed in infancy or early childhood. Other outstanding questions include the number of doses required to optimize responses to the gonococcus both qualitatively and quantitatively, and the potential to affect carriage.

Several lines of evidence suggest a vaccine that could impact on the growing burden of gonorrhea already exists. While the mechanisms are not yet understood, elucidating these will contribute to the ongoing development of high efficacy vaccine options for this disease. In order to successfully deploy a vaccine that could impact on the prevalence of gonorrhea the development of formulations that target all pathogenic *Neisseria* species might be the most socially acceptable, while also the most pragmatic when considering implementation into already crowded immunization schedules.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Proteomics, Bioinformatics and Structure-Function Antigen Mining For Gonorrhea Vaccines

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Expanding efforts to develop preventive gonorrhea vaccines is critical because of the serious health consequences combined with the prevalence and the dire possibility of untreatable gonorrhea. Reverse vaccinology, which includes genome and proteome mining, has proven successful in the discovery of vaccine candidates against many pathogenic bacteria. Here, we describe proteomic applications including comprehensive, quantitative proteomic platforms and immunoproteomics coupled with broad-ranging bioinformatics that have been applied for antigen mining to develop gonorrhea vaccine(s). We further focus on outlining the vaccine candidate decision tree, describe the structure-function of novel proteome-derived antigens as well as ways to gain insights into their roles in the cell envelope, and underscore new lessons learned about the fascinating biology of *Neisseria gonorrhoeae*.

Keywords: *Neisseria gonorrhoeae*, vaccine, proteomics, antigen, reverse vaccinology, bioinformatics, protein structure-function, membrane vesicles

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INTRODUCTION

Worldwide, over 78 million people are estimated to acquire the sexually transmitted infection gonorrhea every year (1). Female reproductive health is disproportionately affected by this disease and half of infected women show no symptoms (2–4). Serious health consequences are associated with untreated or insufficiently treated gonorrhea, including pelvic inflammatory disease, inflammation of the fallopian tubes, pre-term delivery, miscarriage, or ectopic pregnancy (5–7). Additionally, infants born vaginally to infected mothers are exposed to the disease in the birth canal and are thus at risk of developing a sight-threatening conjunctivitis (8). Although the health consequences to men are not as severe as for women and predominantly manifest as uncomplicated urethritis accompanied by a neutrophil-rich exudate (6, 9), gonorrhea can ascend to the epididymis or the testes and may require surgical removal of the infected site (10–12). Infertility can occur in both females and males without proper treatment (9, 13).

The bacterium responsible for gonorrhea, *Neisseria gonorrhoeae* (*Ng*), is a highly adaptable pathogen. Its natural competence and plastic genome have contributed to the extensive spread of antibiotic resistance. Through a number of horizontally acquired genes and chromosomal mutations, *Ng* has become resistant to every antibiotic used for its treatment (14–16). The Centers for Disease Control and Prevention (CDC) in the United States currently recommend a dual therapy of intramuscular ceftriaxone combined with oral azithromycin as a first-line treatment for uncomplicated gonorrhea (17, 18). However, the first isolates resistant to this combination therapy have begun to emerge (19). Three new therapeutics for gonorrhea treatment are being evaluated in clinical trials (20), but considering the speed with which the gonococcus develops antibiotic

resistance (15), new drugs will not provide a long-term solution. The development and introduction of a protective vaccine against gonorrhea should therefore be prioritized to limit its spread.

Thus far, only two gonorrhea vaccines, using either killed whole organisms or purified pilin protein, have progressed to clinical trials. Despite robust antibody responses in both trials, neither vaccine provided protection against acquiring the disease after immunization (21–24). These failures are likely due to a number of factors. Pilin proteins undergo extensive antigenic variation through frequent recombination with transcriptionally silent *pilS* gene cassettes (25–28). Experimental infections have demonstrated that multiple pilin variants are isolated from a single individual, and that these variants are antigenically distinct from the inoculating parent strain (29–31). Further, pilin proteins are subjected to phase variation, where protein expression transitions between "on" and "off" states through slipped-strand repair of upstream repeat regions (32). Antigenic and phase variation of pilin during infection likely contributed to the failure of both vaccine trials. Another factor that may have led to the whole cell vaccine's inability to protect from infection is the presence of the reduction modifiable protein (Rmp; also known as protein III) in the vaccine. Localized to the outer membrane, Rmp is highly conserved and immunogenic, yet antibodies induced by this antigen actively prevent assembly of the complement membrane attack complex in immune serum (33, 34). These challenges illustrated the necessity for new approaches in gonorrhea vaccine development.

In the intervening years, vaccine progress has been slow. One of the difficulties is that *Ng* infection rarely, if ever, leads to an adaptive immune response (35–38). For this reason, mechanisms of protection against gonorrhea are unknown (24), which makes the evaluation of the potential efficacy of vaccine candidates prior to expensive immunization studies challenging. The serum bactericidal activity of antibodies generated during an immune response strongly predicts protection for vaccines against *N. meningitidis* [*Nm*; (39, 40)], a frequent causative agent of meningitis, so the ability of gonococcal antigens to elicit bactericidal antibodies is currently used as a surrogate mechanism of protection (41). Based on this criterion, 14 *Ng* antigens with functions in colonization and invasion, nutrient acquisition, and immune evasion have been proposed for inclusion in a gonorrhea vaccine [reviewed in (41)]. Immunization with each of the candidate proteins, cyclic loop peptides, or lipooligosaccharide epitope mimics elicited bactericidal antibodies, although studies for seven of the antigens were performed only in *Nm* (41).

Despite the difficulties in developing a vaccine against gonorrhea, several recent advances suggest that a protective vaccine is now within reach. The first was the development of a female mouse model of lower genital tract infection, in which mice are treated with 17-β estradiol and a cocktail of antibiotics to increase susceptibility to *Ng* and to reduce the overgrowth of vaginal commensal bacteria, respectively (42). This model has enabled the study of the immune response to gonococcal infection in a whole organism for which extensive genetic and immunological tools are available (24, 43, 44). A series of elegant studies, combining information gathered from experimental murine infections and tissue culture experiments, demonstrated *Ng* actively suppresses the generation of a productive adaptive immune response. Both mouse splenic mononuclear cells and human dendritic cells infected with *Ng* produced elevated levels of interleukin (IL)-6, tumor necrosis factor-α (TNF-α), IL-1β, and IL-23, a set of cytokines that promote terminal differentiation of T-cells toward T helper 17 (Th17) cells (45, 46). Production of IL-17 is a characteristic marker of a Th17 response and promotes neutrophil recruitment through the induction of granulocyte-colony stimulating factor and chemokines (45). In support of gonorrhea promoting Th17 differentiation during an active infection, elevated levels of IL-17 were discovered in female mice challenged with *Ng* (46). Gonococci are also able to divert T-cell differentiation away from an adaptive Th1/Th2 response by inducing the production of transforming growth factor (TGF)-β and IL-10 (47–49). Furthermore, *Ng* stimulates the differentiation of macrophages toward a regulatory phenotype and prevents macrophage antigen display. Through these immunosuppressive activities, the gonococcus is able to further inhibit the generation of a protective T-cell response (50, 51). The knowledge gained through these studies into the sophisticated methods *Ng* uses to promote its own survival and prevent triggering an adaptive immune response will enable a more informed strategy for vaccine development and help avoid the failures of the past.

In studies making use of the insights gathered from a better knowledge of the immune evasion strategies employed by the gonococcus, mice treated intravaginally with micro-encapsulated IL-12 and either infected with a common laboratory strain, FA1090, or immunized with membrane vesicles (MVs) collected from FA1090 were protected against subsequent infections up to 6 months after the initial treatment, even when challenged with heterologous strains (52, 53). IL-12 treatment promoted a Th1 response, as well as enhancing serum immunoglobulin A (IgA) and vaginal IgA and IgG levels (54).

Lessons can also be learned from the successful development of the licensed four-component *Nm* serogroup B vaccine, 4CMenB (BEXSERO; GlaxoSmithKline). This bacterium presented a daunting vaccination challenge for a number of years due to the polysaccharide capsule surrounding group B meningococci, which is structurally identical to the polysialic acid carbohydrate found on the surface of many human cells. Because of this similarity, immunization with the group B capsule is minimally immunogenic and/or may result in the generation of autoantibodies (55). To circumvent this problem, a subunit vaccine was developed by identifying conserved open reading frames in the whole genome sequence of *Nm* serogroup B, a strategy termed reverse vaccinology (55–59). Out of nearly 600 vaccine candidates identified with this strategy, 350 were successfully expressed and purified from *Escherichia coli*, 28 elicited bactericidal antibodies, and only three recombinant proteins—two of which are composed of a fusion of two proteins—were combined with MVs to formulate 4CMenB (59, 60). Finally, a retrospective study found that immunization with another *Nm* serogroup B vaccine, MeNZB, containing the same MVs as 4CMenB, was 31% effective at preventing gonorrhea (61, 62). The MeNZB vaccine is no longer

available, but these seminal studies provide strong evidence that a protective gonorrhea vaccine is possible.

A comparison of the number of antigens evaluated for the serogroup B vaccine with the number currently being investigated for a gonorrhea vaccine illustrates how far gonorrhea research lags behind meningitis research and emphasizes that new strategies are necessary to increase the pool of vaccine candidates under consideration. An innovative way to address this gap was to perform reverse vaccinology antigen mining using subcellular fractionation coupled with high-throughput quantitative proteomics followed by bioinformatics (63, 64), which identified numerous stably expressed proteins and suggested that formulation of a subunit vaccine against gonorrhea would be successful. Both genome- and proteome-based reverse vaccinology approaches have become more prevalent since the technique was introduced (56). Candidate vaccine antigens have been identified through whole-genome screens of a number of medically important pathogens (65–69). As the availability of bacterial genome sequences has increased, more detailed analyses have become possible, including comparative genomics. One weakness of using whole genome sequences to search for vaccine candidate antigens is that the pathogens do not necessarily express the proteins discovered through this approach. Transcriptome analysis provides a way to circumvent this limitation but a low correlation between transcriptomic and proteomic data has been well established [reviewed in (70)]. For this reason, we have chosen to pursue proteomic-based reverse vaccinology, as proteomic studies reveal the biologically relevant population of proteins expressed during exposure to the conditions under examination (63, 64, 71). Proteomic approaches also have the potential to specifically identify surface-exposed proteins without the need for extensive bioinformatic predictions (72).

In this article, we provide an overview of proteomic and bioinformatic approaches that have been utilized for gonorrhea antigen mining. Our focus will also be on functional and structural characterization of proteome-derived antigens to determine their role in gonococcal pathogenesis and physiology as well as to inform the development of next generation vaccines based on structural vaccinology.

PROTEOMIC TECHNOLOGIES APPLIED FOR GONORRHEA ANTIGEN MINING

Surface-localized proteins represent attractive vaccine candidates, as they are important foci for the immune system and play pivotal roles in bacterial physiology as well as host-pathogen interactions. Naturally elaborated MVs (NeMVs) and MVs extracted from cell evelopes (CE) by either lithium or deoxycholate treatment (LeOMVs or DeOMVs, respectively) contain surface-localized proteins, other outer membrane and periplasm-derived proteins, and commonly cytoplasmic proteins (73–79). In addition to proteins, MVs contain lipopolysaccharide and DNA of chromosomal, plasmid, or phage origin, as well as RNA (73, 75, 80). NeMVs are purified and concentrated from culture supernatants by separating intact cells from already-formed NeMVs (**Figure 1**). Le-MVS and De-MVs are extracted from bacterial cells with detergent, reducing the content of reactogenic LPS/LOS and also many lipoproteins (75, 81, 82). Including different types of MVs in vaccine formulations has led to some of the most effective vaccines against bacterial diseases (61, 81, 83). Remarkably, following a nationwide implementation of 4CMenB, a recent study showed >80% vaccine-mediated protection against current *Nm* B strains in the United Kingdom (84, 85).

For all these reasons, the identification of CE and MV antigens is a key objective of proteomics-driven vaccinology. It is however, a difficult task, because membrane proteins are commonly low abundant, hydrophobic, have a basic charge, and can be of high molecular weight. Therefore, comprehensive antigen mining of bacterial CE and MVs necessitates extensive subcellular fractionation procedures coupled with high-throughput quantitative proteomics and extensive bioinformatics (**Figure 1**). In addition, multidimensional protein identification technology combats these challenges by using a combination of two different kinds of liquid chromatography (2D-LC) that separates proteins prior to their identification, greatly diminishing the complexity of the sample at the peptide level and resulting in the identification of a superior number of proteins (75, 86).

Accordingly, to interrogate the *Ng* CE and MVs for new antigens, three independent proteomic technologies and experimental designs have been applied for the first time in the gonorrhea field (52, 63, 64). In the earliest proteomic mining, four common laboratory *Ng* strains (FA1090, MS11, F62, and 1291) were cultured under standard growth conditions in liquid medium to mid-logarithmic phase and subjected to fractionation, sodium carbonate extraction, and ultracentrifugation steps to isolate NeMVs and CE proteins [**Figure 1**; (63)]. Subsequently, these subproteomes were trypsinized and labeled with four different isobaric tags (114, 115, 116, and 117) targeting ε-amine group of lysine in peptides for relative and absolute quantitation (iTRAQ). After labeling, the samples were pooled and the peptide mixture was subjected to fractionation by 2D-LC followed by MS/MS for protein detection and quantitation. This multiplexed high-throughput proteomics approach enabled identification of 533 and 168 common proteins in the CE and MVs, respectively, in all four *Ng* strains in biological replicate experiments. Strain FA1090 was arbitrarily selected as the reference strain for calculating the protein abundance. After applying rigorous criteria, Zielke et al. (63) eliminated up to 68% of identified proteins. Among these proteins, 305 and 46 were uniformly present in the CE and MVs, respectively, in four strains. A total of 22 proteins were present at different levels in both analyzed subproteomes of these strains. Overall, these studies led to identification of a plethora of proteins that were either novel or had not been characterized in *Ng*. In this group were ubiquitous proteins localized to the CE and MVs: LPS-transport protein LptD (OstA, Imp), BamA, BamE, a predicted extracellular protein NGO1063 (SliC), and outer membrane proteins NGO1205 (ZnuD), NGO1344 (AsmA), NGO1956 (TamA), NGO1985 (BamG), NGO2111 (Slam2), NGO2121 (MlaA), NGO2139 (MetQ), and NGO2054 (63, 64).

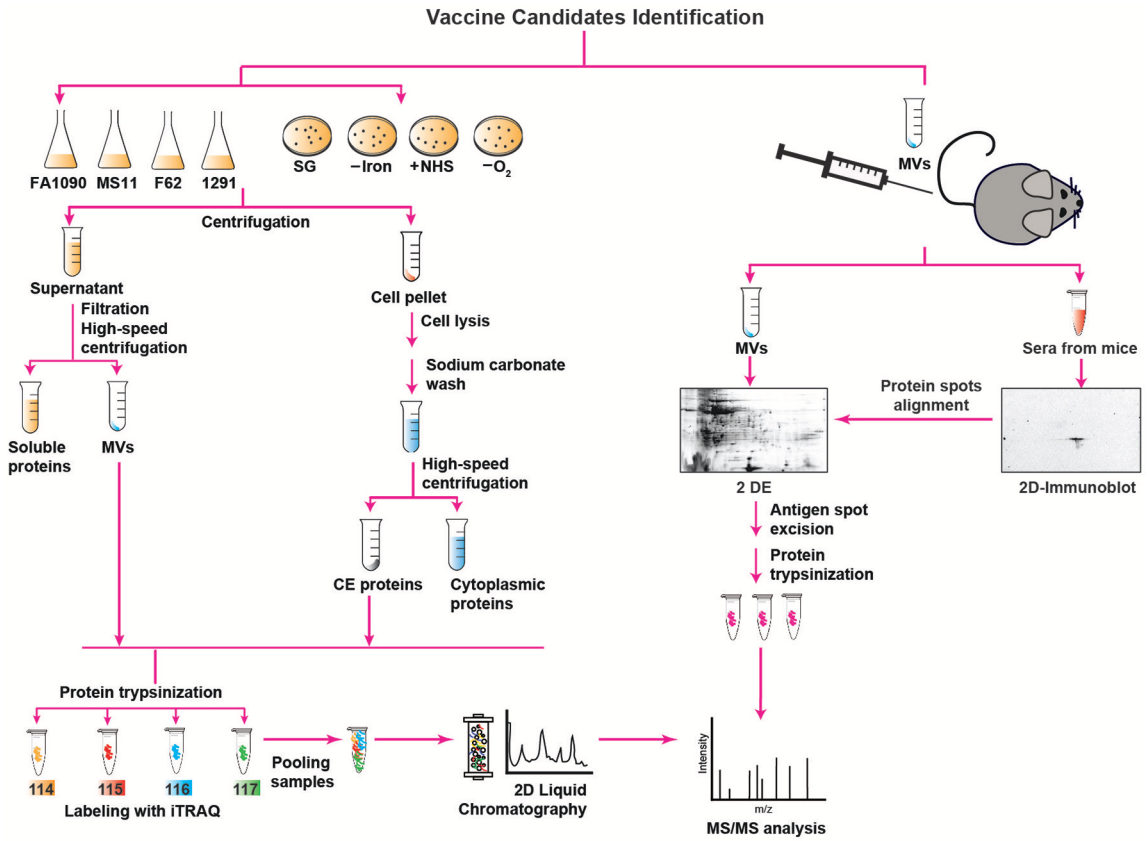

FIGURE 1 | Outline of proteomics approaches used for mining of *Neisseria gonorrhoeae* vaccine antigens. Description is provided in the main text. SG, standard growth conditions; –Iron, iron deprivation; +NHS, addition of normal human serum; $–O_2$, anaerobic growth conditions; MVs, membrane vesicles; CE, CE; 2 D, two-dimensional; 2 DE, two-dimensional electrophoresis; iTRAQ, isobaric tagging for absolute quantitation; MS/MS, mass spectrometry analysis.

We focus on these proteome-derived antigens in the later sections of this article.

In the second proteomics-driven antigen mining approach for gonorrhea vaccine(s), our group was interested in discovering antigens induced in response to host-relevant environmental stimuli as they may represent novel protective antigens in distinct niches in the human host (64). Therefore, the model *Ng* strain FA1090 was subjected to aerobic (SG) and anaerobic ($–O_2$) conditions, iron deprivation (–Iron), and exposure to normal human sera (+NHS; **Figure 1**), followed by CE protein extraction, trypsinization, iTRAQ labeling, and 2D-LC MS/MS (64). Three biological experiments yielded 751 common proteins with 17, 32, and 367 proteins with altered expression compared to SG in the presence of NHS, upon iron deprivation, and during anaerobic growth, respectively. In addition, 259 proteins were ubiquitously expressed under all conditions. There were many newly identified ubiquitous and differentially expressed CE proteins and potential new antigens including Slam2 and NGO1688 (both positively regulated by low iron), and ZnuD, which was induced under oxygen limitation (64).

In addition to the aforementioned approaches, we applied classical immunoproteomics to identify potential cross-reactive antigens in native MVs derived from *Ng* FA1090 that were intravaginally inoculated concurrently with interleukin-12 (**Figure 1**) and showed protection against heterologous *Ng* strains in the female mouse model of lower genital tract infection (52). Our approach relied on 2 DE SDS-PAGE separation of *Ng* native MVs coupled with immunoblotting with sera from MV-immunized mice (**Figure 1**). The overall MV proteome maps were created by staining proteins in a fluorescent stain. After the superimposition of antigenic maps (2D Immunoblot) with proteome maps, matching spots were excised and the proteins were subjected to trypsin digestion and MS/MS-based identification. The blotted protein maps consistently showed two spots of masses corresponding to 45 kDa and 43 kDa that were identified by MS/MS as translation elongation factor-Tu (EF-Tu) and a putative periplasmic polyamine-binding protein, PotF3, respectively. Supporting these findings, both proteins were also identified in our quantitative proteomic profiling of MVs derived from four common gonococcal isolates (63).

BIOINFORMATICS FOR GONORRHEA VACCINES

After the mining and discovery of potential new antigens, insights can be gained into their suitability in a vaccine formulation through bioinformatic analyses to predict their

function, subcellular localization, and post-translational modifications (**Figure 2**). In our studies, we employed PSORTb (87, 88), SOSUI-GramN (89), and CELLO (90, 91) algorithms to infer protein localization within the cell and to identify proteins discovered in the CE or MVs that localized to the outer membrane (63, 64). While each method considers the physiochemical properties of the amino acids that make up the protein, follow up computations differ between algorithms. To increase the accuracy of the subcellular localization predictions in our studies, we utilized a majority-votes strategy in which proteins were assigned to cellular compartments based on the results of at least two of the three methods (63, 64). To provide additional confidence in the localization predictions, searches for signal sequences recognized by signal peptidase (SPase) I or SPaseII can also be performed. The SignalP 4.1 server[1] can be used as a tool to detect signal peptides (SP; **Figure 3**) associated with targeting to secretory pathways and cleaved by SPaseI (92, 93), while the LipoP 1.0 server[2] detects lipoprotein signal peptides (LSP; **Figure 3**) associated with cleavage by SPaseII and further processing through the lipoprotein maturation pathway (94). Lipoproteins are characterized by an invariant cysteine residue that is modified with two or three acyl chains, which allows a hydrophilic protein to remain anchored to the membrane (95). Depending on secondary sorting signals, which are not well understood, lipoproteins may be anchored to either the inner or outer membrane and may face the periplasm or the extracellular milieu. Surface-exposed lipoproteins may be involved in nutrient acquisition, cell wall homeostasis, and adhesion to host cells (95), properties which we will discuss in more detail below. Finally, lipoproteins exposed to the extracellular milieu act as ligands for Toll-like receptor (TLR) 2 and may thus contribute to activating an adaptive immune response (95), a feature which makes them intriguing targets for vaccine development.

An additional analysis that can be performed to complement the prediction of subcellular localization includes functional category predictions through searches for Clusters of Orthologous Groups (COG). This analysis involves a phylogenetic comparison of the proteome-derived vaccine candidates to a database of proteins with known or predicted functions to determine to which cellular process(es) the protein is likely to contribute (96, 97). The results are grouped into four broad categories—Cellular Processes and Signaling, Information Storage and Processing, Metabolism, and Poorly Characterized—each of which can then be broken down further. Altogether, proteins can be clustered into one or more of 25 categories. Although the NCBI COG database[3] does not contain information for *Ng*, COG data is available for *Nm*, which allows for functional analysis using a closely related organism. Insights gained into the function of the proteome-derived vaccine candidates will enable a more rational vaccine design approach, in which candidate antigens involved in multiple functions or in functional categories critical to bacterial fitness can be preferentially chosen in the vaccine decision tree and evaluated for their protective capabilities (**Figure 2**).

The proliferation of genomic data also benefits proteomics-driven reverse vaccinology. Of particular utility to gonorrhea vaccine research is the publically available *Neisseria* Multi Locus Sequence Typing database[4] [*Neisseria* PubMLST, developed by Keith Jolley and sited at the University of Oxford; (98)], which has collected whole or partial genome sequence data from nearly 47,000 *Neisseria* isolates as of August 19, 2018. Annotation of the *Neisseria* pan-genome is underway, which would further facilitate vaccine development by identifying antigens that are present across the known population of *Ng* isolates, as well as proteins that are uniquely associated with highly antibiotic resistant strains. The wealth of information available from this database enables bioinformatic analyses of antigen conservation across numerous sequenced isolates. For example, in our characterization of a novel surface exposed lysozyme inhibitor of C-type lysozyme encoded by the *ngo1063* open reading frame, SliC, use of the *Neisseria* PubMLST database revealed the existence of only 10 closely related alleles with 9 single nucleotide polymorphic sites across nearly 5,000 *Ng* isolates. Further, 98% of isolates in the database possessed a single *sliC* allele (99), indicating that this protein is highly conserved and its inclusion in a vaccine could provide broad protection without the need for multiple antigenic variants.

Another analysis made possible by the information present in the PubMLST database is to map polymorphisms to available structural data (**Figure 2**). If highly prevalent polymorphisms are found on surface-exposed portions of the proteins, multiple recombinant proteins that incorporate the most common variants may need to be included in a vaccine to provide protection against a broader range of strains. While structural data will not necessarily be available immediately for all vaccine candidates, another computational analysis method may still enable prediction of surface exposed polymorphisms. Prediction of transmembrane helices by hidden Markov modeling using the TMHMM 2.0 server[5] can reveal protein regions predicted to be internal, transmembrane, or external (100). Combining data from PubMLST with the results of TMHMM predictions can suggest which polymorphisms are likely to be surface exposed. Importantly, this analysis must be accompanied by surface-exposure assessments, as we discuss below, and may not be accurate for lipoproteins where the lipid anchor could be the only portion of the protein embedded in the membrane.

Additionally relevant to vaccine research are bioinformatic tools designed to predict the immunogenicity and protective capability of candidate antigens. Depending on whether the desired immune response is humoral (B-cell mediated) or cellular (T-cell mediated), numerous tools exist to predict whether antigen-derived peptides possess epitopes that are likely to be recognized by either of the major histocompatibility complex (MHC) class I or II proteins, the transporter associated protein (TAP) responsible for translocating peptides across the endoplasmic reticulum to MHC molecules for surface display, or

[1]http://www.cbs.dtu.dk/services/SignalP/
[2]http://www.cbs.dtu.dk/services/LipoP/
[3]https://www.ncbi.nlm.nih.gov/COG/
[4]https://pubmlst.org/neisseria/
[5]http://www.cbs.dtu.dk/services/TMHMM/

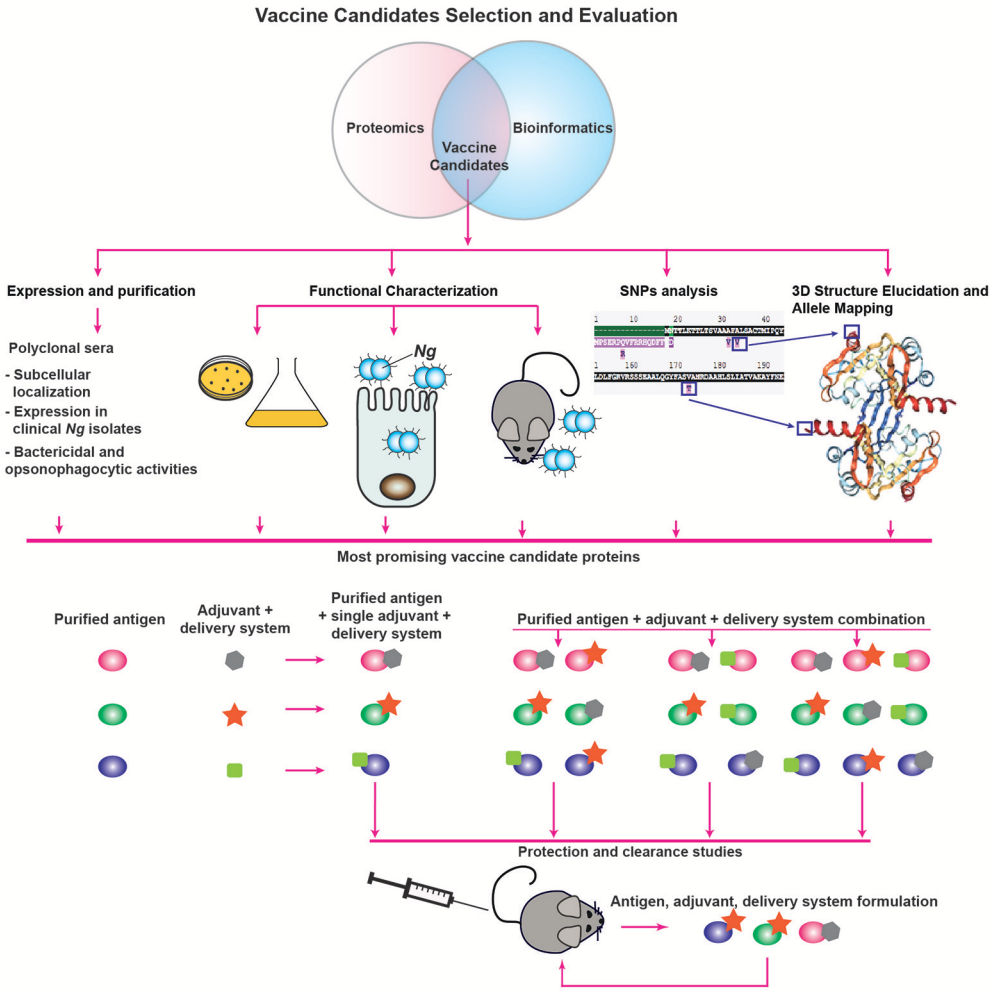

FIGURE 2 | Vaccine candidate decision tree. Description is provided in the main text.

B cell receptors. MHC I predictive tools have been estimated to be 90–95% accurate, although MHC II predictions are less reliable. Structural information is required for accurate B cell receptor binding predictions, as B cells and antibodies recognize the protein's native form. Certain tools use biochemical properties of the protein's constituent amino acids to predict likely B cell receptor binding sites. More accurate techniques require the protein's 3D structure as input [prediction tools extensively reviewed in (101)]. These prediction strategies may be useful to filter out antigens that are not likely to generate an immune response. However, follow-up immunological testing in the mouse as described below will be required to establish whether the response is protective or whether, as in the case of Rmp, the immunogenic response actively blocks the action of the adaptive immune system (33, 34).

VACCINE DECISION TREE

Subsequent to proteomic identification and bioinformatic analysis, candidate antigens should be assessed for their suitability for inclusion in a vaccine. Here, we suggest a decision tree for the evaluation of vaccine candidate proteins (**Figure 2**). To determine the expression characteristics of each vaccine candidate, they should initially be expressed and purified from a heterologous host, such as *E. coli*, and used to immunize rabbits or mice to collect polyclonal immune serum specific to the protein under investigation. These sera can then be used for several informative studies, such as confirmation of localization predictions by probing subcellular fractions to determine in which compartment(s) the protein is predominantly located. Immune sera can be used to interrogate the abundance of proteins after proteolytic shaving of intact cells, an experiment which will demonstrate whether proteins are accessible to external proteases (64). Protein accessibility to antibodies can also be directly investigated by immunoblotting analysis of intact cells spotted onto membranes and comparison of the signal to lysed cells using known surface-exposed proteins as controls (64). Antisera generated against vaccine candidates coupled with fluorescently labeled secondary antibodies can also be used to establish antigenic

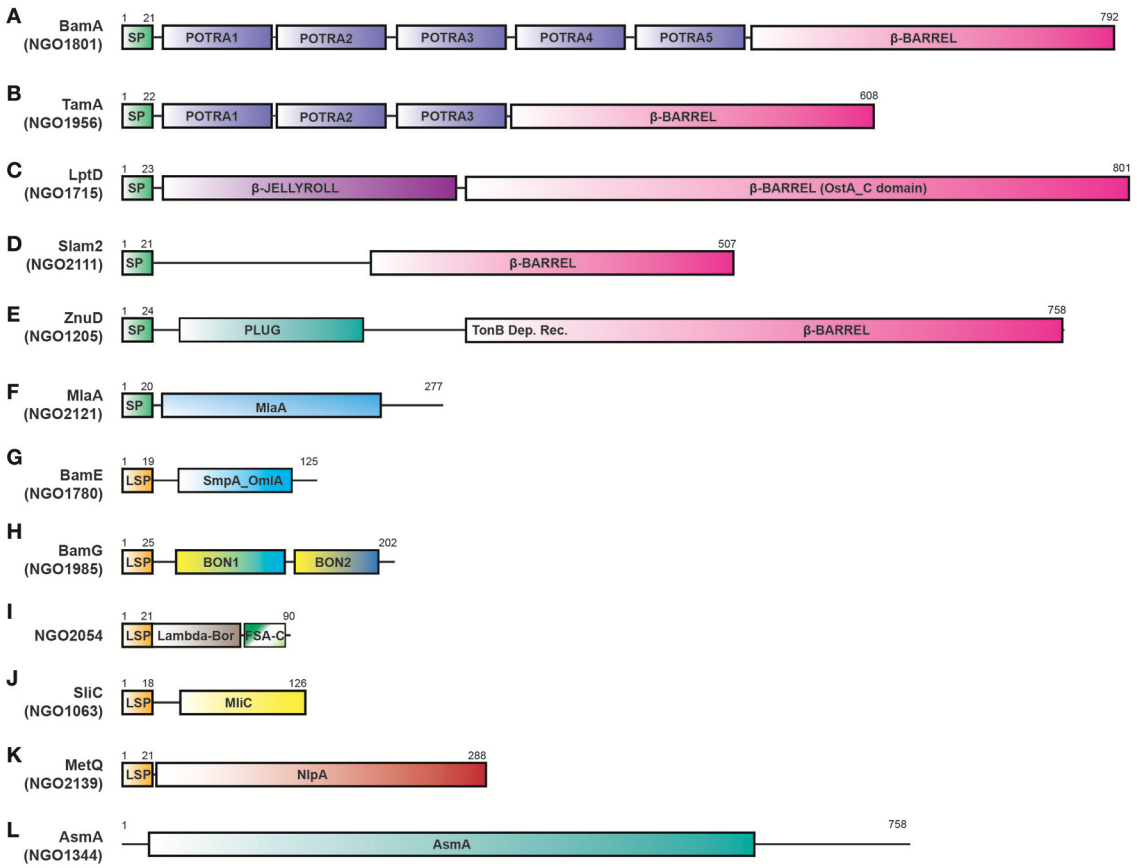

FIGURE 3 | Proteome-derived antigen domain schematics. ORFs were examined for the presence of domains using KEGG, NCBI, UNIPROT, and literature searches. The presence of signal peptides was determined using the SignalP 4.1 and LipoP 1.0 servers. **(A)** BamA with a signal peptide, five POTRA domains, and an OMP β-barrel domain. **(B)** TamA protein with a signal peptide, three POTRA domains and an OMP β-barrel domain. **(C)** LptD with a signal peptide, N terminal β-JELLYROLL domain, and β-barrel OstA_C domain. **(D)** Slam 2 with a signal peptide and C terminal β-barrel domain (a DUF560 domain). **(E)** ZnuD comprises a signal peptide, plug domain, a Ton-B dependent receptor domain, and an OMP β-barrel domain. **(F)** MlaA with a signal peptide and predicted MlaA domain. **(G)** BamE containing a lipoprotein signal peptide and SmpA_OmlA domain. **(H)** BamG is composed of a lipoprotein signal peptide and two BON domains. **(I)** NGO2054 contains a Lambda-Bor domain that overlaps with a lipoprotein signal peptide, as well as a Fragile Site Associated protein C-terminus (FSA-C). **(J)** SliC containing a lipoprotein signal peptide and MliC protein family domain. **(K)** MetQ comprises a lipoprotein signal peptide and a NlpA domain **(L)** AsmA includes an AsmA domain. Schematics are not to scale. SP, signal peptide; LSP, lipoprotein signal peptide; BON, bacterial OsmY and nodulation; FSA-C, Fragile site-associated protein C-terminus; OMP, outer membrane protein.

surface exposure through fluorescence-activated cell sorting analyses (68). Furthermore, primary antisera coupled with gold nanoparticle-labeled secondary antibodies can be employed in electron microscopy studies of surface exposure. With this type of study, not only can surface exposure be confirmed, but the localization, distribution and overall abundance of surface proteins can also be directly observed (102, 103). Serum raised against a candidate antigen additionally enables studies to accompany conservation predictions performed during initial bioinformatic analyses. In our assessments of candidate antigens, we employed a panel of at least 36 genetically, geographically, and temporally distinct *Ng* isolates, including the 2016 WHO reference strains (104), to examine whether the expression of each antigen is consistent across heterogeneous strains, as well as to determine whether the epitope(s) recognized by the antiserum are conserved in diverse gonococci and meningococci (64, 79, 99). Finally, to determine the likelihood that immunization with vaccine candidate antigens will be protective, antiserum raised against each antigen can be used to assess the protein's ability to elicit bactericidal and/or opsonophagocytic antibodies (24, 43). As mentioned previously, it is unknown whether antibodies' bactericidal or opsonophagocytic properties will predict their protective capabilities (24, 41). However, one or both characteristics is likely to contribute to a productive adaptive immune response, so this examination is useful in the absence of established protective mechanisms.

Studies that can be performed in tandem with antiserum-enabled investigations involve characterizations of the antigens' function(s) in gonococcal physiology and pathogenesis (**Figure 2**). We discuss these investigations in greater detail in a later section. In broad terms, the experiments associated with functional characterization are designed to assess the effects

of conditional or isogenic knockout mutations on the ability of *Ng* to thrive *in vitro* under standard conditions as well as during exposure to stimuli relevant to infection, its ability to adhere to and invade epithelial cells, and its ability to colonize female mice during experimental infection in the murine model of lower genital tract infection or transgenic mice (41, 42). The overarching goal of these experiments is to determine the effects of the loss of each vaccine candidate, as antibodies are known to block protein function. In *Ng*, neutralizing antibodies against the nitrite reductase AniA inhibited its enzymatic activity (105). Neutralizing antibodies were also demonstrated to prevent MetQ-mediated gonococcal adhesion to epithelial cells (106), and were able to abrogate *in vitro* lysozyme inhibition by the *Neisserial* adhesin complex protein (ACP), a dual-function protein involved in adhesion and defense against lysozyme attack (99, 107–109). Additionally, *Nm* and *Ng* IgA1 protease-mediated IgA cleavage was inhibited by sera collected from both acute and convalescent meningitis patients (110). More specifically, antibodies that block protein function are elicited by immunization with each of the recombinant protein components of the 4CMenB vaccine—factor H binding protein (fHbp), Neisserial heparin binding antigen (NHBA), and *Neisseria* adhesin A (NadA). Antibodies against fHbp abrogate factor H binding and thus enhance meningococcal serum sensitivity (111). Both NHBA and NadA are involved in bacterial adherence, and neutralizing antibodies against either protein reduce the ability of *Nm* to adhere to epithelial cells (112, 113). Non-neutralizing antibodies provide protection against viruses (114, 115); however, similar data are scarce for bacteria (114). Targeting bacterial virulence factors or physiologically important proteins with a vaccine could discourage mutations that would allow the protein to evade immune detection, thereby improving the vaccine's success. The exclusive focus on targeting virulence factors in a vaccine is not vital, however, as demonstrated by immunization with the *S. agalactiae* surface immunogenic protein (Sip), which induces a strong protective response (116) but has no reported function in the pathogenesis of group B streptococci. It is possible that the antigens eventually formulated into a successful gonorrhea vaccine will not generate neutralizing antibodies, but evidence suggests antibodies that block protein function to some extent are elicited in the majority of immune responses effective at protecting against bacterial pathogens.

An additional study that can be used to inform vaccine design involves antigen structural elucidation, either alone or in complex with antibodies directed against the target protein. We discuss structural studies that have been performed on proteome-derived vaccine candidates in a subsequent section. Not only does the structure of a protein give insights into its function, but it also enables allele mapping, as discussed in the previous bioinformatics section (**Figure 2**). Traditionally, structural characterization is performed through X-ray crystallography (117), although advances in nuclear magnetic resonance spectroscopy also allow for the structural elucidation of small proteins (118, 119). Co-crystallization with antibodies can reveal the mechanism(s) of action of the antibodies against the target protein (120–122). This strategy requires the use of antigen binding fragments of monoclonal antibodies (mAbs), which are more technically challenging, time-consuming, and expensive to produce than polyclonal antibodies (123). For this reason, co-crystallization is likely to be pursued only after further evaluation of the antigen's immunogenicity and protective capabilities as part of a vaccine. A complementary technique that can be pursued for difficult-to-crystallize antigen/antibody complexes is cryo-electron microscopy, in which single molecules embedded in a flash-frozen matrix are visualized with an electron microscope. This technique was successfully employed to elucidate the structure of an integral membrane ion channel protein (124), as well as the interaction interface between an antibody fragment that successfully neutralized a range of influenza virus variants and the receptor site of the influenza virus hemagglutinin protein (125). One limitation of cryo-electron microscopy is that structural resolution tends to be poorer than with the use of other techniques. However, recent technical advances have been able to improve acquired structures to near-atomic resolution [<4Å; (126)]. Although cryo-electron microscopy may circumvent some limitations of X-ray crystallography or nuclear magnetic resonance, mAbs will still be required for evaluating antigen/antibody interactions.

Finally, if the results of the evaluation studies suggest that a protein may be a suitable vaccine component, its immunogenicity and ability to elicit a protective response will be investigated through protection and clearance studies performed in the female mouse model of gonorrhea (**Figure 2**). To date, only an extremely limited number of studies have been published examining the ability of vaccine formulations to accelerate clearance and protect against subsequent infection in the mouse model. These studies include intranasal immunization with MVs, which was associated with enhanced clearance in one study (127), but not in another (24). However, intravaginal immunization with MVs combined with microencapsulated IL-12 both accelerated gonococcal clearance and protected mice from subsequent infection (52). Active intraperitoneal immunization with a peptide mimic of a conserved LOS epitope recognized by the 2C7 mAb or passive immunization with the mAb itself both shortened disease duration (128). Finally, mice immunized in the rear footpads with viral replicon particles (VRPs; viral derivatives that deliver antigens after a single replication cycle) loaded with the outer membrane porin PorB, combined with a subsequent booster immunization comprised of recombinant refolded PorB and an adjuvant, cleared experimental infections significantly more quickly than control mice (24). Follow up experiments suggested that this protection was likely associated with the adjuvant effect of the VRP itself, rather than a specific protective effect of the PorB antigen (24). These studies, and their paucity, reveal the need to accelerate and expand systematic immunization studies that examine the protective effects of antigens individually and in combination. Additionally, the effects of the presence or absence of adjuvants with each antigen formulation should be examined, as different adjuvants can drive specific adaptive immune responses (**Figure 2**). Currently, vaccines that induce a balanced Th1/Th2 responses are considered optimal [reviewed in (129)] and are important in defending against gonorrhea (52, 53).

Examples of FDA-approved adjuvants that should be evaluated based on these considerations include alum, which drives a Th2 response; monophosphoryl lipid A, a detoxified LPS derivative that acts as a TLR4 agonist to stimulate a Th1 response; and oligonucleotides enriched in cytosine phosphoguanine (CpG) islands (129, 130), which are TLR9 agonists that mediate Th1 and CD8 cellular immune responses but also increase antibody titers (131). To optimize the chance of developing an effective vaccine, the route of immunization, as well as different antigen delivery systems that potentiate immune responses, should be considered and investigated. Numerous delivery systems are in clinical and pre-clinical stages of development, including virus-like particles, emulsions, liposomes, polymer-based systems, hydrogels, and implants [reviewed in (129, 130)]. Finally, evaluation of subunit vaccines beyond their ability to accelerate clearance and protect against subsequent infection will include quantification of antigen-specific IgG and IgA antibody titers in serum and vaginal mucosal secretions, evaluation of antibodies' bactericidal and opsonophagocytic activities, and examination of the cellular immune response.

While we recognize that the decision tree presented here may appear daunting, it is important to remember that traditional vaccinology has failed to deliver a successful gonorrhea vaccine. Development of new vaccines is not trivial. The challenges inherent in protecting against this highly adaptable pathogen necessitate creative and flexible approaches. The decision tree and the generation of effective gonorrhea vaccines will be shaped by lessons learned from the *Ng* biology and epidemiology as well as new delivery systems and technologies. A strength of our proposed decision tree is that, regardless of whether an antigen is ultimately formulated into a vaccine, more information will be gained into *Ng* pathophysiology and the gonorrhea research field will be accelerated.

ELUCIDATION OF FUNCTION OF THE PROTEOME-DERIVED VACCINE CANDIDATES

We propose a vaccine-induced immune response that targets antigens important for CE homeostasis, bacterial pathogenesis, or overall viability may not only protect against the acquisition of subsequent gonorrhea infections but may also enhance immune system efficacy by weakening the gonococcus, thus accelerating the clearance of ongoing infections. A vaccine that employs this strategy could conceivably be used for therapeutic interventions in addition to preventative purposes. Studies evaluating the effectiveness of a vaccine against herpes simplex virus type 2 (HSV-2) provide a precedent for a therapeutic vaccine. In both guinea pigs (132) and humans (133, 134), vaccination against HSV-2 tended to decrease the lesion rate and reduced viral shedding for at least 12 months—an evaluation criterion for efficacy of HSV-2 antiviral agents (134). A neutralizing antibody response was key for the vaccine's immunotherapeutic activity (132). For this reason, determining the function of antigens proposed for inclusion in a vaccine is useful to predict the potential physiological effects of an immune response that blocks protein function. The scientific literature contains a wealth of information that can be used as a starting point to facilitate functional characterization. Numerous gonococcal proteins have homologs that have been investigated in *E. coli* or *Nm*. However, these studies should be approached with a modicum of caution, as protein function may differ between species. For example, we determined that LptD is essential in *Ng* (63), similar to findings in *E. coli* (135, 136). In contrast, this protein is dispensable for *Nm* (137). Furthermore, fHbp, which is a surface-exposed protein that contributes to *Nm* serum resistance, has no signal peptide for outer membrane localization in gonococci, nor does loss of *Ng* fHbp alter bacterial susceptibility to human serum (138). The potential for distinct protein function, even among closely related species, emphasizes the importance of performing independent studies to examine the role of homologous proteins in the organism being targeted by the vaccine.

A common tool for the study of protein function is a bacterial strain with a knockout mutation in the protein of interest. This can be accomplished through homologous recombination-mediated allelic replacement of the genetic locus with an antibiotic resistance marker, as we have performed in our studies (63, 64, 99), or through gene inactivation by targeted transposon insertion mutagenesis (139). Of course, attempting to knock out an essential gene with this strategy will be unsuccessful, as transformation efforts will not result in any colonies, or off-target mutations may occur that lead to antibiotic resistance but do not affect the target gene. Neither outcome is desirable. Our strategy to circumvent this difficulty has been to place the gene of interest at an unlinked locus under the control of an inducible promoter, then to replace the native gene with an antibiotic resistance cassette while inducing protein expression from the heterologous locus (64, 140, 141). With this technique, the effects of protein depletion, as well as protein stability, can be studied for essential genes. Using knockout mutant strains of non-essential genes, protein function can be assessed by exposing bacteria to different stress conditions and monitoring for growth. We evaluate bacterial survival under conditions relevant to human infection, including iron starvation, exposure to human serum, and anoxia, with the hypothesis that bacteria deficient in proteins important to a rapid or appropriate response to any of the conditions will be non-viable or will not grow as robustly as wild type bacteria (79, 99, 142). CE permeability and stress can be evaluated by exposure to different antibiotics, either with the use of Etest antimicrobial test strips[6] or serial dilutions of bacteria inoculated onto solid medium supplemented with antibiotics (63, 79, 99). Additionally, overproduction of MVs is a general marker of CE stress (143–145), so a comparison of MV production between wild type and mutant bacteria can suggest the level of CE stress resulting from the loss of a protein (79). Qualitative or quantitative proteomic profiling of supernatants collected from liquid cultures can give insights into the extent of membrane leakage or cellular lysis associated with deficiency of each vaccine candidate (146, 147). Finally, *in vitro* tissue culture experiments can inform whether the protein under investigation is involved in, or has substrates that contribute to, the ability of *Ng*

[6]http://www.biomerieux-usa.com/clinical/etest

to adhere to, invade, and survive within human cervical epithelial cells (99, 106, 148, 149).

An innovative technique that we adapted for the first study of its kind in *Ng* is the use of phenotype microarrays [PMs; (147)]. Developed by Biolog[7], each PM is a 96-well microtiter plate pre-formulated with varying concentrations of numerous diverse compounds to assess bacterial nutritional requirements and sensitivity to chemical agents, including antibiotics and osmolytes (150, 151). *Ng* nutritional requirements are well established. Glucose, pyruvate, and lactate are the only carbon sources the gonococcus is able to utilize (152). Therefore, we focused solely on chemical sensitivity PMs to assess the ability of seven vaccine candidates to defend against osmotic shock, as well as their physiological roles during exposure to CE-perturbing agents including metals, antimicrobial peptides, small hydrophobic molecules, and dyes (147). In our investigation with a comprehensive screen of over 1,000 conditions, we discovered 323 conditions that affected at least one of the mutant strains tested. Using these data, we generated a dendrogram based on the similarity between the effects of the loss of each protein, which revealed that the defects associated with knockout mutations of BamG or MlaA were the most distinct from the other five strains tested. The results of PM screening suggested these two antigens would be the most suitable to include in a vaccine, due to the extensive chemical sensitivities associated with the loss of either protein (147).

Although *in vitro* experiments are useful for predicting protein function, it is impossible to simultaneously account for all factors that will be encountered during infection of the host. For this reason, infection studies in the female mouse model are invaluable (**Figure 2**). Experimental infections with a single strain can be used to determine whether the bacterial load or infection duration is altered in the absence of a target protein (42, 153). Alternatively, competitive infections between the mutant strain and wild type bacteria can be performed to minimize mouse-to-mouse variation and to directly associate fitness phenotypes with the loss of a protein (153). Finally, to determine whether the protein under investigation contributes to bacterial response to hormones, ovariectomized mice can be utilized to decrease hormonal influence over infection characteristics (43, 153).

The studies outlined above will facilitate vaccine development by identifying antigens with roles in maintaining gonococcal fitness under stress conditions, including during active infections.

FUNCTION OF PROTEOMIC-DERIVED ANTIGENS IN CE HOMEOSTASIS AND NUTRIENT ACQUISITION

In the following sections, we discuss insights into function-structure of 12 proteomics-derived antigens (**Figure 3**). We have already verified surface-exposure for a majority of these vaccine candidates, as well as their expression and conservation among diverse *Ng* isolates. We have also established that BamA, TamA, LptD, MetQ, and NGO2054 elicit bactericidal antibodies that cross-react with heterologous *Ng* strains (64). Our initial characterization of these vaccine candidates in *Ng*, in addition to studies of the functions of homologous proteins in *Nm* show that many of them play different roles in the CE homeostasis or in nutrient acquisition. Maintaining the integrity of the OM, as well as acquiring nutrients, are critical for bacterial survival in hostile environments, such as those encountered during infection of the host. As a gram-negative bacterium, *Ng* possesses a typical CE, composed of a cytoplasmic or inner membrane, a cell wall made up of peptidoglycan, and an asymmetric OM (**Figure 4**). The outer membrane of *Neisseria spp* contains LOS, rather than the more typical LPS, which reflects their niche as mucosal pathogens (154). LOS acts as a buffer region for protection against environmental insult and contributes to pathogenesis through several mechanisms (154, 155).

Three of our antigens (BamA, BamE, and BamG) are components of the β-barrel assembly machinery (BAM) system (63, 79, 156), which is responsible for folding and inserting β-barrel proteins into the bacterial outer membrane (157, 158). Until recently this critical protein complex has been primarily investigated in *E. coli*, where it consists of the central β-barrel "Omp85" protein BamA, assisted by four lipoproteins (BamB-E). In contrast to *E. coli*, gonococcal BAM lacks BamB and contains a surface-displayed BamE [**Figure 4**; (79)]. Further, *Neisseria* possess an additional non-essential accessory protein RmpM (159). Similarly to other gram-negative bacteria, BamA and BamD are essential for *Ng* cell viability, and depletion of either BAM component results in OMP misfolding, as well as defects in protein stability and assembly (64, 160–162). BamA is responsible for folding and inserting virtually all β-barrel OMPs in the OM (158). In contrast, BamE is not essential, which enables study of its effects on the CE. Our comprehensive analyses, including PMs, showed that the loss of BamE renders *Ng* susceptible to a wide range of compounds including detergents, antimicrobial peptides, other membrane-perturbing agents, and antibiotics (79, 147). Enhanced production of MVs is a marker of CE stress (143). Accordingly, the Δ*bamE* mutant produced significantly increased amounts of MVs in comparison to WT bacteria. Additionally, the MV protein profile exhibited distinct alterations, suggesting that certain proteins were specifically targeted for packaging into MVs (79). Finally, removal of BamE appeared to destabilize the BamA-D interaction, which resulted in BamD localized on the cell surface of BamE-deprived *Ng* (79). The aberrant BamD localization in the absence of BamE could further increase membrane perturbations by interference with proper OMP insertion by BamA.

We recently discovered that one of our proteome-derived vaccine candidates, NGO1985, is a previously unrecognized accessory lipoprotein within the BAM complex and therefore renamed this protein BamG (156). Included in BamG is a lipoprotein signal peptide, which targets the protein for surface display (156), as well as two bacterial OsmY and nodulation (BON) domains (**Figure 3H**). BON domains are proposed to bind phospholipids based on the finding that *E. coli* OsmY prevents the inner membrane from shrinking during osmotic

[7]https://biolog.com/products-portfolio-overview/phenotype-microarrays-for-microbial-cells/

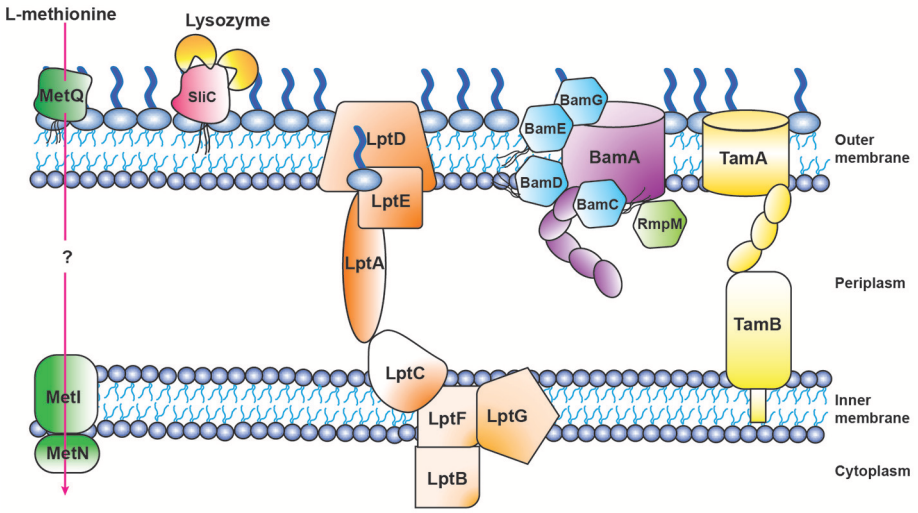

FIGURE 4 | Schematic of proteomics-derived antigens MetQ, SliC, LptD, BAM, and TAM function-structure and organization in *Neisseria gonorrhoeae*.

shock, although this hypothesis was not experimentally verified (163–165). In *Ng*, our initial probing with detergents and PM analyses revealed an extensive sensitivity phenome associated with lack of BamG, suggesting that BamG, through its interaction with the BAM complex, plays a critical function in CE biogenesis (63, 147, 156).

LptD belongs to the low abundant, large and complex class of BAM substrates that are also heavily dependent on SurA, Skp and FkpA chaperones (166). LptD, together with OM-localized LptE (**Figure 4**), are constituents of the lipopolysaccharide transport (LptA-F) complex, and are crucial for inserting LPS into the outer leaflet of the *E. coli* outer membrane (167). The Lpt system appears not to function in a completely conserved manner in gram-negative bacteria, as both *Ng* and *E. coli* LptD are essential (63), whereas *Nm* can survive without LOS and none of the Lpt components are important for bacterial viability (168).

For the bacterial CE to exert its barrier function, the outer leaflet of the outer membrane must be a homogeneous layer of LOS. If this LOS layer is disrupted, phospholipids may diffuse from the inner leaflet and weaken the barrier function of the outer membrane. The Mla (maintenance of lipid asymmetry) system, which is conserved across gram-negative bacteria and is composed of MlaA-F, removes misplaced phospholipids from the outer leaflet and re-integrates them into the inner membrane to mitigate the detrimental effects of altered membrane asymmetry. The outer membrane component of the Mla system, MlaA, has primarily been studied in *E. coli*, where its deletion enhances bacterial susceptibility to membrane perturbing agents (169). In *Ng*, MlaA is encoded by the *ngo2121* locus and comprises a signal peptide and a MlaA domain (**Figure 3F**). Gonococcal MlaA is annotated as a lipoprotein, although we have previously noted that it does not contain the invariant cysteine residue required for lipid modification (147). However, a lipoprotein signal peptide is present in homologs from *E. coli, K. pneumoniae,* and *S. marcescens* (169, 170). We are currently investigating the implications of the lack of MlaA lipidation in *Ng*. Combining antibiotic susceptibility testing using Etests with PM screening, we have established that compounds targeting the CE, including antimicrobial peptides, are more effective against *Ng* lacking MlaA than wild type bacteria. Resistance to antibiotics acting against cytoplasmic targets was unaltered (63, 147, 171), suggesting a specific outer membrane defect (169). Additionally, the Δ*mlaA* knockout showed increased vulnerability to oxidative stress-inducing compounds (147) and produced more MVs than wild type bacteria (171). Together, these findings suggest that MlaA is required for maintenance of outer membrane homeostasis and is involved in MV biogenesis (172).

Bacterial lipoproteins play numerous roles in cellular physiology, adhesion to host cells, modulation of inflammatory processes, and transport of virulence factors into host cells. Proper lipoprotein localization is critical for protein function (173). The proteome-derived vaccine candidates BamE, BamG, SliC, MetQ, and NGO2054 are newly described *Ng* lipoproteins (**Figure 3**). NGO2111 is homologous to the *Nm* surface lipoprotein assembly modulator Slam2, which is involved in translocating the hemoglobin-haptoglobin utilization protein to the cell surface (174). Slam2 is highly conserved amongst *Neisseria* isolates and is not found in *E. coli* (175, 176). *Ng* Slam2 contains a signal peptide and a 14-stranded β-barrel domain, which has also been annotated as a DUF560 domain (**Figure 3D**; 147, 176). In our PM study, we found no conditions that uniquely affected a Δ*slam2* mutant, although 36 compounds were either beneficial or detrimental to the growth of this strain, in common with one or more of 6 other mutants (147). These results suggested that Slam2 exerts an indirect effect on CE integrity, potentially through an undiscovered lipoprotein substrate.

Another gonorrhea vaccine candidate we investigated in our PM study was NGO1344, which contains an AsmA domain (**Figure 3L**) and is homologous to the AsmA protein from *E. coli* and *Nm*. In *E. coli*, loss of AsmA decreased LPS synthesis

(177); however, a similar phenotype was not observed in a *Nm* Δ*asmA* mutant (178). Our analysis of cell lysis indicated that loss of *Ng* AsmA resulted in elevated levels of several cytoplasmic proteins in culture supernatants, especially when grown in the chemically-defined Graver-Wade liquid medium (147). Fifty-three membrane-perturbing compounds affected the viability of a Δ*asmA* mutant, including polymyxin B and bile salts (63, 147). Clustering analyses suggested that the loss of AsmA resulted in phenotypes similar to those observed upon BamE deletion (147). Our results indicate that gonococcal AsmA contributes to CE integrity.

We also examined the role of the small lipoprotein NGO2054 in CE homeostasis. This protein is comprised of a lipoprotein signal peptide, a Lambda-Bor motif that contributes to serum resistance in *E. coli* (179), and a region with homology to the C-terminus of the fragile site-associated protein involved in adipocyte differentiation in mammalian cells [**Figure 3I**; (180)]. A Δ*ngo2054* mutant was the least affected in our PM screen; only 27 compounds altered its growth (147). Thus, although we have established that NGO2054 is surface exposed, well conserved, and elicits bactericidal antibodies (64), its function within the CE remains enigmatic.

Metal co-factors such as zinc and iron are critical to facilitate cellular and enzymatic processes within pathogenic bacteria. During infection, bacteria rely on scavenging these metals from the host. To combat against bacterial pathogens, host organisms sequester available metals at the site of infection, a strategy termed nutritional immunity. The *Neisserial* outer membrane zinc uptake protein ZnuD overcomes host-imposed zinc depletion (181). In *Ng*, ZnuD is encoded by the *ngo1205* locus, which includes an amino terminal signal peptide, a plug domain, as well as a TonB β-barrel (**Figure 3E**). In support of its role as a zinc uptake protein, a *Nm* Δ*znuD* mutant was more sensitive to the bactericidal effects of neutrophil extracellular traps (182), which contain calprotectin that sequesters zinc ions (183). Additionally, meningococci deficient in ZnuD were attenuated in a systemic infection model (181), although Δ*znuD* gonococci exhibited no survival defect during intracellular infection of cervical epithelial cells (184). We performed our PM analysis on *Ng* Δ*znuD* bacteria to examine the contribution of ZnuD to CE integrity. Thirty-seven conditions altered the growth of bacteria lacking ZnuD, including the divalent cation chelator ethylenediaminetetraacetic acid (147). Our results provide support for the role of ZnuD as a zinc uptake protein and suggest that downstream effects to the CE occur from the decreased ability to acquire zinc.

Translocation of solutes across the CE is mediated by ABC transporters, which utilize the hydrolysis of ATP to transport molecules. A substrate binding protein is necessary to capture the substrate. Our proteome-derived vaccine candidate MetQ (63, 64), which was highly conserved in *Ng* as well as *Nm* (55, 64), is homologous to the *E. coli* methionine binding protein MetQ [also known as NlpA; (106)] and is annotated with a lipoprotein signal peptide and a NlpA domain (**Figure 3K**). The presence of an operon upstream from *metQ*, composed of an ATP-binding protein (MetN) and a transmembrane permease (MetI) (106), provides additional support for the role of MetQ as the substrate binding protein in an ABC transport system. Surface plasmon resonance experiments demonstrated that *Ng* MetQ binds L-methionine with nanomolar affinity (106). These findings, in combination with our experiments demonstrating that *Ng* MetQ is surface exposed, highly conserved, and elicits strongly bactericidal antibodies (64), suggest MetQ is a promising vaccine candidate. Targeting MetQ could interfere with methionine transport and result in downstream protein synthesis defects.

The studies described here illustrate the myriad cellular processes in which our proteome-derived vaccine candidates participate. A subunit vaccine composed of several of these antigens has the potential to compromise gonococcal fitness independent of factors beyond a neutralizing antibody response.

FUNCTION OF SLIC AND METQ IN PATHOGENESIS

Bacterial pathogenicity is influenced by the number of infecting bacteria, the route of entry, the presence of host defense barriers, and bacterial virulence factors. *Ng* pathogenesis involves a collection of factors: pili, Opa proteins, LOS, and peptidoglycan all contribute to infection and are important for optimal pathogenesis (6). The study of protein function has the potential to reveal previously unrecognized virulence factors. A perfect example is our discovery of the surface-exposed inhibitor of C-type lysozyme, SliC (99). Through bioinformatic analyses, we determined SliC, encoded by the *ngo1063* locus, contained a lipoprotein signal peptide and a domain similar to membrane-bound lysozyme inhibitors of C-type lysozyme (MliC domain; **Figure 3J**). Bacterial proteinaceous lysozyme inhibitors protect the cell wall against host lysozyme attack during infection (185). Lysozyme inhibitor proteins were not known to exist in *Neisseria* until the recent discovery that the *Neisserial* adhesin complex protein (ACP) is inhibitory toward human (HL) and hen egg white lysozyme (HEWL), both of which are C-type lysozymes (108). The activity of SliC as a lysozyme inhibitor was comprehensively examined using an assay that involved fluorescently labeled peptidoglycan. Pre-incubation of wild type SliC with HL or HEWL completely obstructed peptidoglycan hydrolysis. In contrast, SliC mutated in two residues predicted to be key for the protein's interaction with lysozyme (S83A and K103A) showed no inhibition of cell wall hydrolysis with the addition of HL or HEWL (99). Subsequently, bio-layer interferometry was used to assess the kinetic interaction between SliC and lysozyme. This analysis revealed moderate binding between SliC and HL *in vitro* (K_D 11 μM), with one SliC molecule binding two HL molecules [**Figure 4**; (99)]. Surprisingly, cells that lacked SliC showed no difference to wild type when exposed to increasing levels of HL *in vitro*. However, a double SliC/ACP knockout was strongly attenuated during HL exposure (99, 109), suggesting that one lysozyme inhibitor compensates for the lack of the other protein. Despite this compensatory activity, *Ng* bacteria lacking SliC were up to 250-fold less fit during a competitive infection with WT bacteria in the mouse model (99). Further, bacteria complemented with a SliC S83A/K103A mutant were also significantly attenuated during competitive

infections (99). *In vitro* adhesion and invasion assays revealed no difference between Δ*sliC* and WT bacteria, which indicated that the attenuation observed in the mouse was not due to defects in the mutant's ability to adhere to or invade epithelial cells. Finally, Δ*sliC* bacteria were as fit as WT during competitive infections in a lysozyme-deficient mouse, which provided conclusive evidence that the inhibitory activity of SliC against host lysozyme is critical for gonococcal colonization (99).

Substrate binding components of ABC transporters may be localized to the outer surface and may contribute to cell adherence (186, 187). MetQ from *Ng* was therefore assessed for its ability to mediate bacterial adhesion and invasion of cervical epithelial cells. Gonococci lacking MetQ exhibited a 2.4-fold decrease in adherence and a 1.5-fold lower level of invasion compared to the wild type strain. These findings were comparable to the level of adherence and invasion within transformed primary cervical epithelial cells (106). Additionally, bacterial survival in primary monocytes and macrophages was evaluated, to determine whether MetQ exerts a protective role against immune cells or other similar factors. Cells lacking MetQ displayed a 2.3-fold reduction of viability in primary monocytes and a 1.5-fold decline in macrophages. MetQ-deficient gonococci were also significantly attenuated upon exposure to human serum (106). The results of this study indicated MetQ, in addition to its role as a methionine transport protein, contributes to the ability of *Ng* to adhere to and invade epithelial cells and protects against the innate immune system.

The study of vaccine candidates' contributions to bacterial pathogenesis benefits vaccine development by revealing potential vulnerabilities that can be exploited through rational vaccine design to cripple the invading bacterial pathogen.

STRUCTURE OF PROTEOMIC-DERIVED ANTIGENS

Structure-based antigen design offers new possibilities in vaccine development and improvement by delivering novel immunogens and informing about protective epitopes (188). This approach, in combination with sequencing data and computational biology studies (189), can drive rational optimization of vaccines as we discussed above (**Figure 2**). In this section, we will focus on summarizing structural investigations that have been performed on our proteome-derived vaccine candidates. To this end, only the structures of *Ng* BamA and BamE have been solved (79, 190). However, *Nm* crystal structures of MetQ and ZnuD are available in addition to LptD, BamA, BamE, and TamA obtained from different bacterial species, all of which provide information about antigen architecture and conformational conservation.

The first BamA crystal structure was solved from *Ng* and revealed a 16-strand β-barrel domain within the outer membrane, connected to five N-terminal periplasmic polypeptide transport-associated (POTRA) domains [**Figure 3A**; (190)]. The BamA crystal structure from *E. coli* displayed a high level of flexibility between $POTRA_5$ and the β-barrel domain, suggesting that the POTRA domains assist in transferring the substrate to the β-barrel domain of BamA (191). A prominent difference was found in BamA conformation in *Ng* compared to BamA in *Haemophilus ducreyi*, where the last β-strands within the barrel are tightly meshed with hydrogen bonds, providing more rigidity. However, in *Ng*, the last β-strand is bound to the first by only two hydrogen bonds, which allows the pore itself to twist. The $POTRA_5$ domain of BamA in *Ng* is positioned closely to the barrel, with periplasmic loops 3, 4, 5, and 7 stabilizing the closed conformation. This is highly different in *H. ducreyi*, as the POTRA region hinges outwards ∼70°, which does not allow $POTRA_5$ to interact with the β-barrel periplasmic loops (190). The difference in the interaction between $POTRA_5$ and the β-barrel may act as a secondary mechanism to prevent unregulated solute entry into the pore and may compensate for the lack of hydrogen bonding in *Ng* BamA. Further analysis of the *Ng* BamA crystal structure revealed the hydrophobic belt along the C-terminal strand was narrower (∼9Å) than the opposite side of the barrel [∼20 Å; (190)]. The authors of this study hypothesize that this reduced width may disrupt the lipid membrane environment and act to allow easier insertion of the OMP into the membrane (190). Based on the configuration of BamA, two hypotheses have been proposed for the mechanism of OMP folding and insertion, depending on the complexity of the substrate protein. Complex proteins take advantage of a lateral opening event facilitated by a conformational switch of loop 6 and the gating motion of the POTRA domains. The nascent OMP is threaded through the β-barrel and uses exposed strands of BamA as a template for proper barrel formation through a transient OMP-BamA complex until the new OMP buds off into the OM. Simple substrates may bypass the BamA β-barrel completely and may be inserted directly into the destabilized portions of the membrane through their interactions with the POTRA domains (190). Recent studies suggest that OMP binding to BamD induces conformational changes in the extracellular loops of BamA for substrate folding and membrane insertion (192). Thus, antibodies against these extracellular loops may interfere with proper OM biogenesis and cause severe downstream effects to OM integrity.

Gonococcal BamE includes a lipoprotein signal peptide, as well as a predicted SmpA_OmlA domain [**Figure 3G**; (193)] In *E. coli*, X-ray crystallography showed BamE interacts exclusively with BamA, and does not contact other accessory lipoproteins. Instead, BamE directly assists OMP folding through its interaction with BamA. In contrast to *E. coli*, where the native form of BamE appears to be a periplasmic monomer (194), *Ng* BamE is a surface-exposed dimer that also includes an additional C-terminal helix not present in other solved structures of BamE (79). Isolation of proteins from native membranes should definitively establish which conformation is the active state. The dimeric form of BamE possesses structural homology to β-lactamase inhibitors, which has led to the hypothesis that BamE has a secondary function as a β-lactamase inhibitor (195, 196). Surface-exposed dimers of BamE in *Ng* may therefore act as a first line of defense against β-lactam antimicrobials. In support of this secondary function of BamE, *Ng* Δ*bamE* mutants were more sensitive to several different β-lactam antimicrobials (79, 147). Further investigation will be required to determine whether BamE contributes to antibiotic resistance. If BamE does in fact

possess dual functions, a neutralizing immune response could both interfere with OM integrity as well as enhance antibacterial efficacy.

Autotransporter biogenesis relies on a passenger-transport complex to assist in the translocation of autotransporters across the outer membrane. The translocation and assembly module (TAM) comprises a two-membrane complex containing a β-barrel OMP TamA and inner membrane protein TamB, a nanomachine required for virulence of pathogenic bacteria [**Figure 4**; (197)]. This complex facilitates the folding and insertion of the autotransporter domain and has been hypothesized to assist in proper effector domain folding. Effector domains are responsible for protein activity and frequently contribute to virulence (198). Consistent with its role in translocation, TamA contains a β-barrel structure homologous to BamA (199) and has been hypothesized to be the result of a duplication event that arose from the evolutionary convergence of BamA and TamB (200). In *E.coli*, the TamA structure was determined at 2.25 Å resolution, revealing a sixteen stranded β-barrel ring structure from amino acids 265-577 on the C-terminus, and three POTRA domains between amino acids 22-264 on the N-terminus [**Figure 3**; (201)]. The POTRA domains wind ∼50Å, oriented toward the periplasm in a semi-circle arrangement (201). The initial contact between substrate and TamA is facilitated by interactions between TamB and the POTRA domains, which act as a hinge mechanism (197, 200). Additional crystal structure analysis showed a kink in the C-terminal β-strand pointing inward, which weakened the lateral wall, implying a possible gate for substrates to route toward the lipid bilayer (201). The kink resulted in a weak β-strand pair of strand 1 and 16, because of three main chain hydrogen bonds. The kink on β-strand 16 results in a large gap between the ends of strands 1 and 15 near the $POTRA_3$ attachment site. This cleft promotes insertion of the substrate, mediated by interactions between the β-barrel and the POTRA domains. The folding and insertion process is hypothesized to begin when an autotransporter's β-barrel domain engages with $POTRA_3$ within the periplasm and is guided toward the barrel of TamA. The structure of TamA also includes a closed extracellular lid from amino acids 456-495. Salt bridge interactions between the lid and the β-barrel are mediated by Arg477 in the lid and Asp521 in the barrel (201). Targeting the epitopes responsible for stabilizing the interaction between lid and β-barrel with an immune response could interfere with proper TamA function.

The crystal structures of the core LPS assembly LptDE protein complexes from several bacteria, including medically important pathogens, have been obtained, with only two full-length structures of *Shigella flexneri* and *Klebsiella pneumoniae* LptDE (167, 202). Overall, these studies revealed a strong structural conservation of the two-protein plug and barrel assembly and demonstrated that LptE was integrated within the 26-stranded, C-shaped β-barrel architecture of LptD. All LptDE structures have a negatively charged lumen which may facilitate LPS/LOS insertion (202). Additionally, LptD contains a periplasmic β-jellyroll domain, which is structurally similar to LptA and facilitates LPS/LOS transit through the periplasm [**Figure 3C**; (167)]. The N terminal domain of LptD undergoes a 21° rotation, which may aid assembly or influence flexibility of the LptCAD scaffold (202). The LptD β-barrel is large compared to other β-barrel OMPs. Its dimensions accommodate large substrates and facilitate efficient transport. Further, a kink was present in the first two β-strands of the β-barrel due to two proline residues in β1 and β2 (167), and they have been experimentally verified to play a pivotal function in the lateral opening of the barrel (202). The interaction between LptD and LptE is dependent on polar connections within the LptD β-barrel inner cavity; thus, LptE supports LptD structural maintenance, as well as export of LPS to the outer leaflet. Disruption of the interaction between LptD and LptE would be catastrophic to proper Lpt complex function (167). Due to the significance of LOS, immune system interference with LptD, and thus LOS transport, would severely alter gonococcal CE composition, weakening the bacterium and reducing its pathogenic capabilities.

The crystal structure of the outer membrane component of the MlaA-F system, MlaA, was recently attained from X-ray crystallography of MlaA proteins from *Serratia marcescens* and *K. pneumoniae*. MlaA is a monomeric α-helical OMP. Its six amphipathic α-helices facilitate the transport of polar phospholipid headgroups while residing within the hydrophobic interior of the outer membrane. The structure of MlaA allows diffusion of outer leaflet phospholipids exclusively (170). It is unclear how the phospholipid is transferred to MlaC, as the MlaA pore does not appear to permit the passage of acyl chains. Interactions between MlaA and MlaC have been hypothesized to induce a conformational change in MlaA, potentially by shifting helix 6 through a gate opening mechanism to enable phospholipid transfer to occur (170). *K. pneumoniae* and *S. marcescens* MlaA proteins were found in complex with the outer membrane pore OmpF in a 3:3 or a 1:3 ratio, respectively (170). The MlaA-porin interplay, mediated by van der Waals interactions, does not appear to significantly influence porin, although the presence of the porin appears to prevent MlaA aggregation (170). Normal function is based on proper architecture of the interaction and linkage of these two proteins. Targeting the maintenance of OM lipid asymmetry through a vaccine could sensitize bacteria to components of the immune system that target the outer membrane.

Neisserial MetQ was originally believed to bind to D-methionine (203, 204). However, structural elucidation from *Nm* revealed L-methionine in the binding pocket, which could not be displaced, even when the protein was heterologously expressed in *E. coli* in minimal medium containing only D-methionine. In support of these findings, and in contrast to the protein's annotation as a D-methionine binding protein, concrete data indicate that D-amino acids are not incorporated into proteins during ribosomal synthesis; L-amino acids are required (205). Biologically, aminoacyl-tRNA synthetases distinguish cognate L-amino acids against noncognate proteinogenic L-amino acids and also nonproteinogenic D-amino acids, thus regulating components for protein biosynthesis (206). However, *E. coli* MetQ is able to bind both D- and L-enantiomers with high affinity (207). Structural comparisons revealed similarities between *Nm* MetQ and L-methionine binding protein Tp32 from *Treponema pallidum* as well as the dipeptide GlyMet-binding

protein Pg110 from *Staphylococcus aureus*, despite low sequence similarity (204). *Nm* MetQ is made up of 15 alpha helices and 10 β-strands, split into two domains, I and II, corresponding to residues 43-119 and 236-281; and 120-235, respectively. The two domains are connected by a hinge region and display a Venus flytrap-like structure, which is typical of periplasmic substrate binding proteins. The methionine binding site is within the crevasse of these two globular lobes (204). Residues critical for proper methionine coordination within the binding pocket include Arg156, which forms a salt bridge with the carboxyl group of L-methionine; Asn215, which forms one of two hydrogen bonds to the nitrogen of L-methionine; and Asn 238, which forms the second hydrogen bond and appears to be responsible for the stereospecificity of *Neisserial* MetQ (204). MetQ is a surface-displayed lipoprotein (64, 106) and thus it remains to be elucidated how L-methinonine is transported through the periplasm to the inner membrane (**Figure 4**).

The structure of ZnuD, a member of the TonB-dependent receptor (TbdR) family, was first crystalized in *Nm* by single isomorphous replacement, combined with opposing signals for native and seleno-derived ZnuD crystals (181). The crystal structure of ZnuD revealed a 22-stranded β-barrel pore architecture similar to that of siderophore domains common to the TbdR family, which includes the amino-terminal plug domain between residues 1-147 and its pore-forming domain from residues 148-734 on the carboxyl terminus [**Figure 3E**; (181)]. The plug domain of ZnuD is required for normal function of TonB in the inner membrane. In contrast to most TbdR proteins, which are involved in iron and cobalamin uptake, ZnuD was the first zinc TbdR structure solved (208). Due to the importance of metal co-factor acquisition, other gram-negative bacteria like *E.coli* use ZnuD ABC transporters to overcome zinc-deficient conditions (209). A key feature of meningococcal ZnuD is the extracellular loops. These loops are arranged in a way that displays a "comb" scaffold, allowing the uptake of zinc. Substrate binding can induce multiple conformational changes that are reversed upon zinc release (181). Key residues assisting in coordination of the zinc ion are Asp99, His100, Glu340 and His499. All four residues are highly conserved within the binding pocket across three intermediate structures (native ZnuD, ZnuD co-crystallized with cadmium, and ZnuD soaked with zinc). The binding pocket is sealed through interactions between the apical loop of the plug and extracellular loop 6. Due to the flexibility of ZnuD, remodeling of the alpha helices and β-strands occurs throughout different binding states. Zinc transport through the β-barrel channel usually requires TonB activation (181). Molecular dynamic simulations found TonB exclusively interacts with the plug domain, which is unfolded upon TonB activation (181). Further investigation using X-ray absorption spectroscopy was performed to determine whether ZnuD specifically binds zinc, or if it interacts with heme as well (181). Superimposition of the ZnuD crystal framework with the hemophore receptor HasR found a similar "lock-key" feature, although ZnuD was determined not to be a heme uptake protein (181, 210). Importantly, meningococcal ZnuD stimulates a bactericidal antibody response that recognizes peptides 233-309, 430-459, and 706-722. These peptides correspond to extracellular loops 3, 6, and 11. Loop 3 is the most immunogenic and performs a pivotal role in blocking access to the zinc binding pocket. A secondary feature of loop 3 is that it shifts to a rigid β-strand conformation upon zinc binding and back to a flexible alpha helix when the substrate is released (181). Despite the significant *Nm* ZnuD conformational changes observed upon zinc binding, its ability to elicit bactericidal antibodies suggests it may be an appropriate vaccine candidate for gonorrhea as well.

The structural studies we have discussed here not only give context to the function of our proteome-derived vaccine candidates, but also give insights into critical, surface-exposed portions of the proteins that can be targeted through structural vaccinology approaches.

CONCLUDING REMARKS

- Proteomics-driven vaccinology for gonorrhea has begun to deliver novel antigens and determined the contents of NeMVs from four different *Ng* isolates, which can further inform the vaccine development and manufacturing processes.
- Proteomics-driven antigen discovery should be paired with comprehensive bioinformatic analyses to enable more informed decisions for rational development of subunit vaccines and facilitate the inclusion of highly conserved surface-exposed proteins with important functions.
- Our proposed approach to vaccine candidate evaluation may facilitate development of the most effective vaccine against gonorrhea in a systematic and cost-effective way suitable for an academic setting.
- The proteomics-derived antigens described participate in essential CE processes as well as pathogenesis. A subunit vaccine composed of several of these antigens has the potential to severely compromise *Ng* fitness.
- Our discovery of SliC as a previously uncharacterized virulence factor illustrates that new lessons can still be learned about *Ng* biology and also highlights the importance of considering that infection occurs in a living host and involves numerous elements that cannot be replicated *in vitro*.
- Structural vaccinology for gonorrhea is in its infancy and thus enhanced efforts should be dedicated to solving structures of all potential vaccine candidates.

AUTHOR CONTRIBUTIONS

FM, BB, and AS wrote the manuscript. BB and AS made final edits. AS provided illustrations.

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Targeting Lipooligosaccharide (LOS) for a Gonococcal Vaccine

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The increasing incidence of gonorrhea worldwide and the global spread of multidrug-resistant strains of *Neisseria gonorrhoeae*, constitute a public health emergency. With dwindling antibiotic treatment options, there is an urgent need to develop safe and effective vaccines. Gonococcal lipooligosaccharides (LOSs) are potential vaccine candidates because they are densely represented on the bacterial surface and are readily accessible as targets of adaptive immunity. Less well-understood is whether LOSs evoke protective immune responses. Although gonococcal LOS-derived oligosaccharides (OSs) are major immune targets, often they undergo phase variation, a feature that seemingly makes LOS less desirable as a vaccine candidate. However, the identification of a gonococcal LOS-derived OS epitope, called 2C7, that is: (i) a broadly expressed gonococcal antigenic target in human infection; (ii) a virulence determinant, that is maintained by the gonococcus and (iii) a critical requirement for gonococcal colonization in the experimental setting, circumvents its limitation as a potential vaccine candidate imposed by phase variation. Difficulties in purifying structurally intact OSs from LOSs led to "conversion" of the 2C7 epitope into a peptide mimic that elicited cross-reactive IgG anti-OS antibodies that also possess complement-dependent bactericidal activity against gonococci. Mice immunized with the 2C7 peptide mimic clear vaginal colonization more rapidly and reduce gonococcal burdens. 2C7 vaccine satisfies criteria that are desirable in a gonococcal vaccine candidate: broad representation of the antigenic target, service as a virulence determinant that is also critical for organism survival *in vivo* and elicitation of broadly cross-reactive IgG bactericidal antibodies when used as an immunogen.

Keywords: *N. gonorrhoeae*, Lipooligosaccharide, vaccine, peptide mimic, complement

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INTRODUCTION

Gonococcal vaccine development is challenging because the correlates of immune protection are not fully known (1); mechanisms of protective immunity against gonococcal infection in humans are also unknown. Potential mechanisms focus on: (1) antibody (Ab) binding to *Neisseria gonorrhoeae* (*Ng*) together with complement activation that results in direct killing (bactericidal activity) of the organism (2, 3); (2) Ab binding and complement activation to enable opsonophagocytic killing (2); (3) Ab binding to prevent adhesion or invasion (4) and (4) T cell help. Beneficial T_H1 responses predominate in several successful vaccine approaches that use a female mouse model of gonococcal infection (5–7).

Gonococcal surface molecules that may be appropriate vaccine targets often are antigenically variable and modify epitopes by antigenic or phase variation (8), which complicates vaccine development by creating an ever-changing bacterial surface. The ability to modify surface determinants is beneficial for gonococci and results in evasion strategies to increase fitness and facilitate adaptation of organisms to their environment. Unfortunately, in human infection, adaptive immune responses directed against conserved antigens fail to elicit protection against future bouts of infection; in fact, repeat infections are common, not only because of re-exposure to unidentified infected partners (9) but also because robust protective immune responses are not elicited. Immune responses that do occur may contain subversive elements that enhance the risk for future infection (10, 11). In female mice, experimental gonococcal infection can suppress the development of adaptive immune responses by inducing regulatory cytokines TGF-β and IL-10 and type 1 regulatory T (Treg) cells (12). Intravaginal treatment of infected mice with IL-12 induces persistent immunity against gonococcal reinfection, which is dependent on the production of IFN-γ and antibodies (13) that results in an enhanced T_H1 response, accelerates clearance of infection and elicits a memory response that results in protection (14).

A number of gonococcal surface components that elicit bactericidal antibodies are under examination as vaccine candidates [reviewed and tabulated (15)]. Immunization with gonococcal outer membrane elicits diverse vaginal and serum antibodies, which can be bactericidal and accelerate clearance of experimental infection (16); however, this approach is not always reproducible (5). An alternative successful approach that favored a T_H1 response, employed mice immunized (primed) with PorB (the gonococcal major outer membrane protein)-expressing Venezuelan equine encephalitis (VEE) virus replicon particles (VRPs), followed by boosting with recombinant Por B (rrPorB) (5, 17). However, elicited antibodies were non-bactericidal.

Several promising vaccine candidates do not elicit bactericidal antibody activity in natural infection but were predicted to be potential vaccine candidates because a more robust immune response may be forced by vaccination that does not occur in natural infection. In addition, bactericidal antibody responses to several antigens may target important physiologic functions that, if disrupted, could compromise *N. gonorrhoeae* further, including colonization and invasion (4, 18–27), nutrient acquisition (28–35), and immune evasion (36–42). Vaccine candidates that elicit bactericidal antibodies have also been identified by proteomic analysis of *N. gonorrhoeae* surface proteins (43) and, for example, by bioinformatic analysis, in *N. gonorrhoeae*, of an adhesin complex protein (ACP) homolog, originally identified in *N. meningitidis* (4). Other vaccine candidates that target function but are not known to elicit bactericidal activity are also discussed in two reviews (44, 45). A recent study surrounding the epidemic of group B *N. meningitidis* infection in New Zealand calculated cross-protective efficacy of 31% against gonorrhea in persons, aged 15–30, who were administered a Group B meningococcal outer membrane vesicle (OMV) vaccine (46), which subsequently has formed the basis of a licensed Group B meningococcal vaccine. Human vaccination with the licensed vaccine elicits antibodies against *N. gonorrhoeae* (47) but they are non-bactericidal (48).

A successful vaccine candidate(s) may exhibit: i) a broadly representative antigenic target(s); ii) a virulence determinant(s) (for example a determinant(s) that facilitates host evasion) that can be neutralized and iii) a determinant(s) that is critical for gonococcal survival. Such a "triple threat" candidate may prove to be a useful strategy to "corner" a skillful organism that employs numerous mechanisms to escape selective pressure. Successful single antigens used as vaccines against bacteria are the capsular polysaccharides (49–52). While these are not present in *N. gonorrhoeae*, saccharide determinants are present in gonococcal lipooligosaccharides (LOSs).

LIPOOLIGOSACCHARIDE (LOS) STRUCTURE

Gonococcal LOSs consist of three oligosaccharide (OS) chains, attached to a lipid A core. The OS chains branch from two heptose residues attached to lipid A via two 2-keto-3-deoxy-mannooctulosonic acid (KDO) molecules. One OS chain elongates from the first heptose (Hep I); the 2nd and 3rd chains are connected to the second heptose (Hep II) (**Figure 1**). The number of branches and the length of OSs in each branch vary among gonococcal strains and, indeed, in the same strain during growth *in vitro* and *in vivo*. The *rfaC* gene that encodes heptosyl transferase is required for the addition of Hep I to KDO (53) (*rfa* genes encoding heptosyl transferases are blocked in yellow in **Figure 1**).

The *rfaF* gene product, also a (different) heptosyl transferase, adds Hep II to Hep I and is required for elongation (schematically depicted as outward in **Figure 1**) of the Hep I chain (54, 55). The synthesis of OS chains is modulated by a series of LOS glycosyl transferases (products of *lgt* genes). *lgtF*, *lgtE, lgtA, lgtB*, and *lgtD* genes are required for stepwise addition of each hexose [or hexosamine in the case of *lgtA* and *lgtD* (shown in **Figure 1**)] to extend the Hep I chain (**Figure 1**) (56, 57). In place of full extension, the *lgtC* gene encodes α-galactosyl transferase that terminates Hep l with galactose (Gal), that can undergo sialylation (shown as Neu5Ac in the orange boxes in **Figure 1**), creating a shorter chain (Galα1-4Galβ1-4Glcβ1-4-) attached to -Hep I (also called the P^K-like LOS) (58). Expression of distinct LOS structures on the gonococcal surface is controlled by the phase variable expression of the LOS glycosyltransferases genes *lgtG, lgtA, lgtC*, and *lgtD* (54, 59) (indicated in red in **Figure 1**). These genes (*lgtA, lgtC*, and *lgtD*) contain homopolymeric tracts of guanine poly (G), and in the *lgtG* gene, a cytosine poly (C) tract (56, 59–62). Slipped strand mispairing during DNA replication can result in alteration in coding sequences, which leads to premature termination of the corresponding genes and loss of function of the encoded glycosyl transferase proteins resulting in truncated LOS structures. Phase variation of LOS results in changes in size of the predominant LOS structures that are expressed both *in vitro* and *in vivo*. LOS undergoes phase variation at a frequency of 10^{-2}-10^{-3} when gonococci are grown in culture (63, 64). Identification of

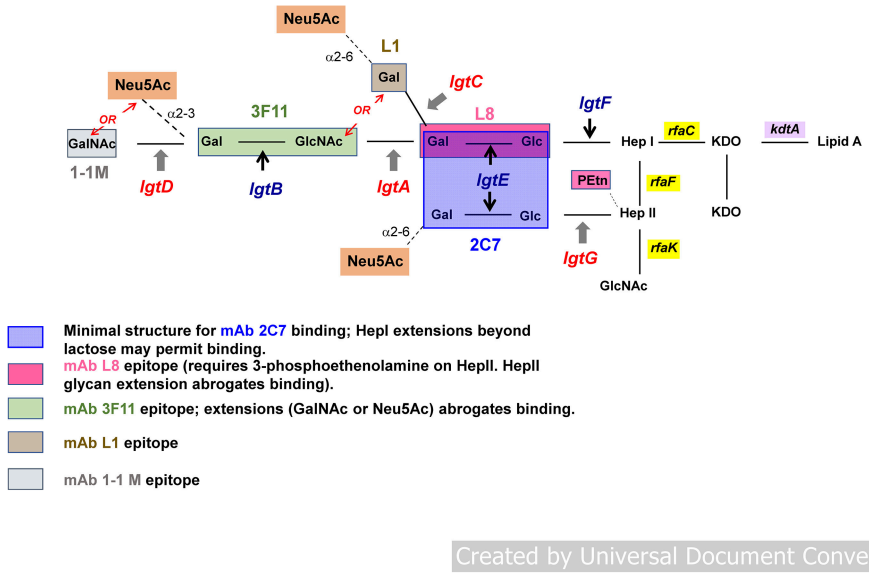

FIGURE 1 | General structure of gonococcal lipooligosaccharide (LOS). Gonococcal LOS consists of three oligosaccharide (OS) chains. The OS chains branch from two heptose residues attached to lipid A via two 2-keto-3-deoxy-mannooctulosonic acid (KDO) molecules. One OS chain elongates from the first heptose (Hep I) outward; two chains extend from the second heptose (Hep II). Lacto-*N*-neotetraose structure (Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-4-) or P^K (Galα1-4Galβ1-4Glcβ1-4-) extend outward from Hep I. Phase variable genes involved in LOS biosynthesis (*lgtA, C, D, and G*) are shown in red; non-variable genes (*lgtF*, *lgtE, and B*) in blue. Neu5Ac (sialic acid) is shown in the orange boxes. Sialylation of LOS occurs via α2-6 or α2-3 linkage to galactose (Gal) residues. LOS branching is terminated ("capped") either by Neu5Ac (sialic acid) or otherwise extend(s) outward by adding hexose(s). LOS epitopes are defined by mAbs 2C7, L8, 3F11, L1, and 1-1M.

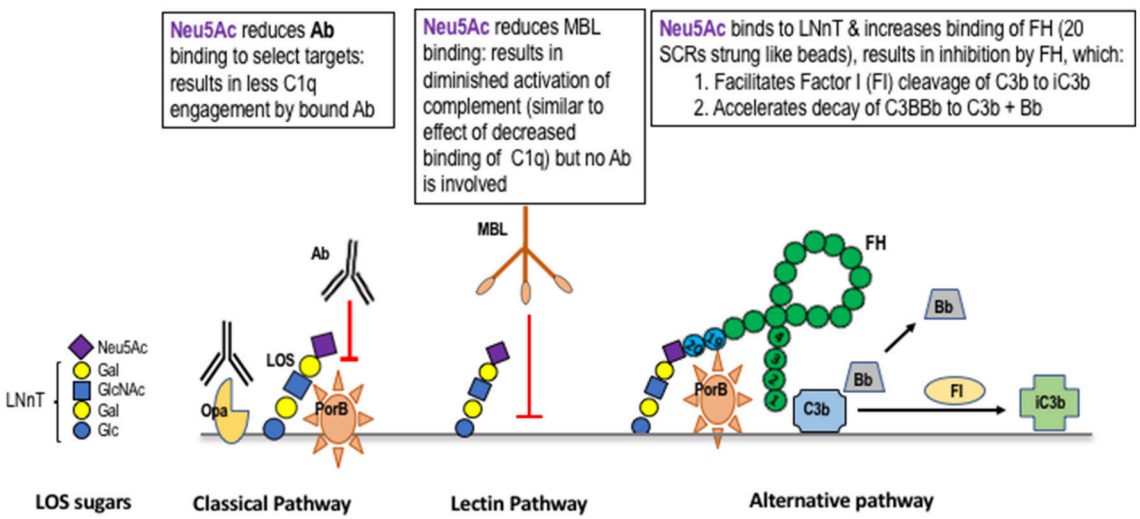

FIGURE 2 | Mechanisms of complement regulation by sialic acid on gonococcal lacto-*N*-neotetraose (LNnT). Gonococci can add N-acetylneuraminic acid (Neu5Ac; the form of sialic acid found in humans) to the terminal Gal of the lacto-*N*-neotetraose (LNnT) LOS structure (a schematic of sialylated LNnT is shown on the left side of the Figure). The presence of Neu5Ac on LNnT LOS reduces the binding of IgG to select targets. As an example, binding of mAbs to PorB, but not to Opacity protein (Opa), is inhibited (79). Sialylation of LNnT also inhibits binding of "natural" IgG in NHS to the gonococcal surface (80). Binding of mannan binding lectin (MBL) to the surface of gonococci is inhibited by LNnT LOS sialylation (81). MBL binds to gonococcal LOS that terminates in GlcNAc (82), which is elongated ("capped") by Gal and Neu5Ac (shown on the left side of the Figure) and to Opa and PorB (83) [Opa and PorB (shown in the Classical Pathway frame to the left of the Lectin Pathway frame in the Figure)]. Neu5Ac that caps LNnT also regulates the alternative pathway of complement by enhancing binding of factor H (FH; shown as a "string of beads" in the Alternative Pathway frame) (40). Enhanced FH binding to sialylated gonococci is restricted to the LNnT structure; sialylation of the P^K-like LOS (84), or lactose on HepII (78) does not enhance FH binding. Binding of FH is also dependent on expression of PorB (85) and occurs through the C-terminal domains of FH (SCR18-20) (86). Bound FH acts as a cofactor in the factor I (FI) cleavage of C3b to iC3b (cofactor activity) and also irreversibly dissociates the C3 convertase, C3bBb (decay accelerating activity).

several of these individual structures on the surface of *Ng* can be demonstrated by reactivity with LOS-specific mouse monoclonal antibodies (mAbs) (depicted by colored boxes in **Figure 1** and in the legend). Several of the antigenic determinants share structure with human glycosphingolipids (GSLs) (58, 65, 66). The lacto-*N*-neotetraose structure (four sugars extending from -Hep I: [Galβ1-4GlclNAcb1-3Galβ1-4Glcβ1-4-]) recognized by mAb 3F11, is identical to human erythrocyte GSLs (67–69). The alternative Hep I structure, digalactoside (Galα1-4Galβ1-4Glcβ1-4-, the P^K structure or the L1 meningococcal serotype (**Figure 1**), is recognized by mAb L1 and is similar in structure to human paraglobosides (58). The fully extended Hep I, a pentasaccharide (GalNAcβ1-3Galβ1-4GlclNAcβ1-3Galβ1-4Glcβ1-4-), defined by mAb 1-1-M (70, 71), has a structure identical to human asialo-G3 ganglioside (**Figure 1**) (69). This mimicry may enable *Ng* to avoid immune recognition; antigenic determinants that share structure with human GSLs, therefore, may not be suitable to elicit a response that is specific for the organism, nor would a response to shared human antigens be desirable.

Nevertheless, *Ng* LOSs possess two epitopes, which do not cross react with human GSL antigens. The first comprises Galβ1-4Glc (lactose), the first two hexoses that are β-linked to Hep I (72–74). Together with a phosphoethanolamine (PEtn) substitution at the 3- (cyclic) position on Hep II (as occurs when *lgtG* is OFF and Hep II is not substituted at the 3-position with glucose [Glc]), this structure is recognized by mAb L8 (75). The second epitope is a composite of the first epitope (L8) plus a Galβ1-4Glc (also lactose) that is α-linked to Hep II (area shaded in blue in **Figure 1**) and represents the minimal structure [*N*-linked fatty acids in lipid A are required for maximal expression (66)] of the epitope recognized by mAb 2C7 (66, 76), called the 2C7 epitope. Absence of Hep II linked lactose (and therefore the complete 2C7 structure/epitope) severely attenuates gonococcal infection in the mouse cervico/vaginal colonization model (7, 77, 78). 2C7 expression therefore, may be an important virulence factor that enhances or may be required for survival and productive infection in humans. Despite phase variation of the *lgtG* gene, that initiates production of the 2C7 epitope (expression is completed by *lgtE*, which is constitutively expressed and adds Gal to Hep II linked Glc) the epitope is widely shared and expressed by most gonococci including 95% of minimally passaged *Ng* clinical isolates (Boston) (2) and in 100% of isolates in Nanjing, China (78). In Nanjing, female subjects who were exposed and infected with *Ng* developed significantly higher levels of 2C7 Ab compared with control women who possessed minimal or no measurable 2C7 Ab. Furthermore, as expected, there was no difference in 3F11 (a self-antigen) antibody levels in infected women vs. controls; neither were there differences in L8 antibody levels between the two groups, all of whom possessed either minimal or no measurable antibody to 3F11 and L8 epitopes. The 2C7 epitope, therefore, is immunogenic in natural infection, more so than at least two other LOS structures that have been antigenically defined.

LOS SIALYLATION AND COMPLEMENT RESISTANCE

Gonococci "cap" LOS molecules in which Hep I terminates with the lacto-*N*-neotetraose structure (four sugars extending outward from Hep I [the LNnT structure]; **Figure 1**). Sialylation can occur using the organism's own endogenous sialyltransferase and appropriate sialic acid substrate(s) present in the mammalian genital tract. *In vitro* (exogenous) cytidine monophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac) serves as a suitable substrate. Sialylated gonococci are endowed with several means to enhance pathogenicity. Sialylation of gonococcal LOS inhibits all three pathways of complement through several independent mechanisms: the classical pathway is inhibited by reducing antibody binding and possibly by reducing C1q (the first component of complement) engagement by bound antibody; the lectin pathway is inhibited by reducing mannose binding lectin (MBL) binding; the alternative pathway is inhibited by increased binding of FH, a major soluble down-regulator in the alternative pathway (**Figure 2**). Sialylation of gonococcal LOS also decreases opsonic killing of gonococci (87–89) in part, because of decreased complement activation and C3 fragment deposition on the surface of sialylated bacteria (38, 40). Sialylation of gonococcal LOS markedly reduces opacity-associated protein (Opa)-mediated invasion of *N. gonorrhoeae* into human epithelial cell lines (90–92). Finally, sialylation of LNnT LOS occurs in organisms present in infected male urethral secretions [by electron microscopy (93)]. The importance of LNnT sialylation for virulence in humans was demonstrated in the experimental model of human infection that used a variant strain of *N. gonorrhoeae* that *in vitro* expressed predominantly Hep I linked lactose (Galβ1-4Glc; L8) but upon recovery from active infection, the sialylatable LNnT species predominated (65, 94). The terminal Gal of the P^K-like structure from Hep I can also be sialylated (84) and recently, Hep II lactose has been shown to accept sialic acid (**Figure 1**) (78), which also inhibits complement deposition and engages Siglec (sialic acid-binding immunoglobulin-type lectin) receptors to down-regulate the host inflammatory response, thereby facilitating host immune evasion (95). Sialylation of the Hep II-attached lactose component of the 2C7 structure/epitope and sialylation (78) may contribute to gonococcal virulence provided by sialylation. Of note, mAb 2C7 continues to bind to *Ng* LOS even when the Hep I chain is extended beyond the minimal lactose structure (66), including binding to sialylated LNnT but less so when the P^K structure/epitope is expressed (96). Glycan extensions beyond lactose on Hep II, for example with GalNAc-Gal seen in a mutant strain selected under pyocin pressure called JW31R, abrogated mAb 2C7 binding (66). However, sialylation of Hep II lactose (78) variably affects binding of mAb 2C7 to gonococcal strains (78). Gonococcal strains that express the P^K structure/epitope are rare/absent *in vivo* (80, 97). Hep II extension beyond lactose, to our knowledge, has not been identified in strains isolated from humans, however, the recently identified additional acceptor site for sialic acid on Hep II lactose (78), suggests that strains bearing sialic acid at this site are likely to be present *in vivo*.

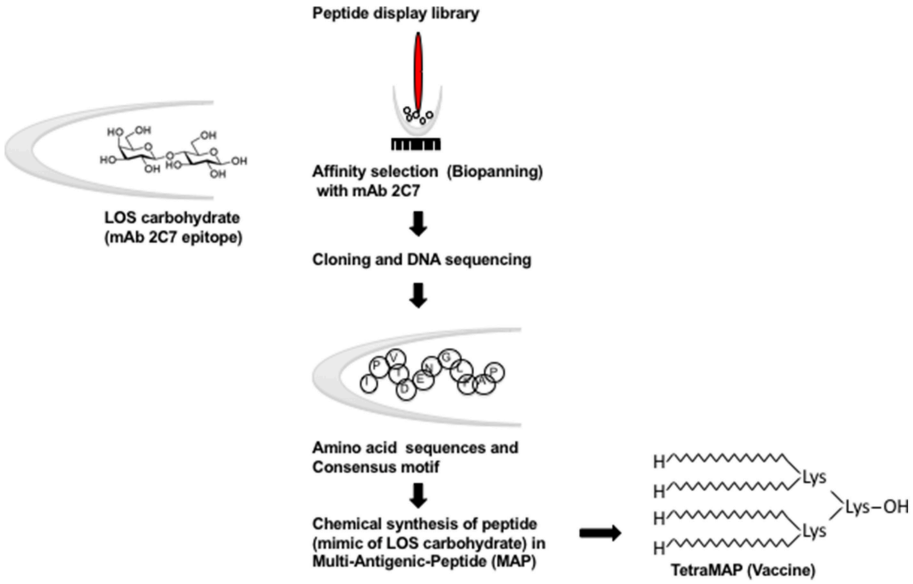

FIGURE 3 | Schematic representation of the conversion of carbohydrate (OS) epitopes into peptide mimotopes. Peptide mimics of the 2C7 epitope were identified using a random FliTrx (Flagellin-Thioredoxin) peptide display library that were screened (Biopanned) using mAb 2C7 (98). The peptide–containing clones that bound to mAb 2C7 were eluted and sequenced. An optimal peptide that contained the consensus motif was synthesized as an octameric peptide (shown here as a multiantigenic peptide [MAP]) on a lysine backbone (TetraMAP), which is the currently used configuration.

THE 2C7 EPITOPE AND ITS PEPTIDE MIMIC

The 2C7 OS epitope has been examined as a potential gonococcal candidate. Carbohydrate (OS) immunogens, themselves, evoke thymus-independent (TI) responses; they stimulate the production of low affinity IgM antibodies predominantly and there is no affinity maturation. Purification of OS from LOS may result in a change in configuration and thereby modify immunogenicity. Because the precise configurations of OS structures within intact LOSs are not known, synthesis would be difficult and optimizing the production of the correct isomers may not be possible without advance structural knowledge. The conversion of carbohydrate (OS) epitopes into peptide mimotopes having similar configuration (defined by recognition of the appropriate mAb, e.g., mAb 2C7 in the case of *N. gonorrhoeae*) is a means to overcome the TI nature of carbohydrate antigens (**Figure 3**). Peptide mimics of the 2C7 epitope were identified using a peptide display library that was screened using mAb 2C7 [a monoclonal Ab with complement-dependent bactericidal and opsonophagocytic activities (2)] and identified peptide mimics were down-selected immunochemically and for immunogenicity (98). Carbohydrates may contain multiple identical antigenic epitopes that provides a molecular configuration allowing carbohydrate to cross-link antigen to their cognate receptors on B cells. To emulate such configurations, an optimal peptide mimotope was chosen and a multiple antigen peptide (MAP) synthesized (**Figure 3**). Immunization of mice with peptide vaccine elicited cross-reactive anti-LOS antibodies that possessed dose responsive direct complement dependent bactericidal activity against gonococci (98). More recent refinements of the peptide building block have been directed: at stabilization to ensure homogeneity; optimization of synthesis to produce high yields and pairing of peptide vaccine with adjuvants that have been approved and used for human vaccination. Further characterization of vaccine induced immune responses evoked by the 2C7 peptide were enlisted to correlate efficacy of active vaccination with MAP in mice followed by experimental vaginal challenge with *Ng*. Mice immunized with MAP combined with monophosphoryl lipid A (MPL), a toll-like receptor 4 (TLR4) agonist, elicited a predominant complement-activating IgG subclass (IgG2a) response resulting from T_H1-biased immune stimulation (7), similar to other vaccine strategies that have proved efficacious in the experimental murine model of *Ng* vaginal/cervical colonization (3, 5). Clearance of *Ng* infection was hastened in vaccinated mice and reduction of bacterial burdens occurred throughout the period of colonization (7). The level of vaccine induced 2C7 immune antibodies in the vaginas of mice correlated directly with reduction in bacterial burden (80). Results of active immunization with the peptide mimic were paralleled by similar results obtained with passive immunization of mAb 2C7 (7). These results strongly support a vaccine antibody-mediated effect that was dependent on the presence of local IgG antibody in mouse vaginas (80). 2C7 vaccine satisfies the three criteria proposed above for a gonococcal vaccine: (i) similar antigenic target representation across strains; (ii) a representative virulence determinant and (iii) a critical determinant for organism survival *in vivo*.

CONCLUSION

Evidence that gonococcal vaccination can succeed in humans is encouraging. Although field trials with whole cell and pilus vaccines have been unsuccessful (99, 100), this occurred, in part, because of exposure of vaccine recipients to heterologous strains in the wild, different than were used to prepare vaccines. Homologous protection in human experimental infection was also shown to be possible in men with favorable antibody ratios directed against the strain used in experimental infection suggesting that protective immunity against broadly cross-reactive antigens will be necessary (15) while avoiding subversive effects that might otherwise undermine protective immune responses (101). Adaptation of such an antigen(s) could result in a successful vaccine. Recent epidemiologic evidence indicates that cross-reactivity between *N. meningitidis* and *N. gonorrhoeae* antigens induces a measurable level of cross protection (46), fulfilling, perhaps, the "triple threat" criteria indicated above that also applies to 2C7 vaccine: (i) broad representation; (ii) service as a virulence determinant and (iii) a critical role in organism survival.

AUTHOR CONTRIBUTIONS

SG and JS organized and prepared material for this manuscript. SR and PR contributed in the writing and reviewing of the manuscript.

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Utility of Hybrid Transferrin Binding Protein Antigens for Protection Against Pathogenic Neisseria Species

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The surface transferrin receptor proteins from *Neisseria gonorrhoeae* have been recognized as ideal vaccine targets due to their critical role in survival in the human male genitourinary tract. Recombinant forms of the surface lipoprotein component of the receptor, transferrin binding protein B (TbpB), can be readily produced at high levels in the *Escherichia coli* cytoplasm and is suitable for commercial vaccine production. In contrast, the integral outer membrane protein, transferrin binding protein A (TbpA), is produced at relatively low levels in the outer membrane and requires detergents for solubilization and stabilization, processes not favorable for commercial applications. Capitalizing on the core β-barrel structural feature common to the lipoprotein and integral outer membrane protein we engineered the lipoprotein as a scaffold for displaying conserved surface epitopes from TbpA. A stable version of the C-terminal domain of TbpB was prepared by replacing four larger exposed variable loops with short linking peptide regions. Four surface regions from the plug and barrel domains of *Neisseria* TbpA were transplanted onto this TbpB C-lobe scaffold, generating stable hybrid antigens. Antisera generated in mice and rabbits against the hybrid antigens recognized TbpA at the surface of *Neisseria meningitidis* and inhibited transferrin-dependent growth at levels comparable or better than antisera directed against the native TbpA protein. Two of the engineered hybrid antigens each elicited a TbpA-specific bactericidal antibody response comparable to that induced by TbpA. A hybrid antigen generated using a foreign scaffold (TbpB from the pig pathogen *Haemophilus parasuis)* displaying neisserial TbpA loop 10 was evaluated in a model of lower genital tract colonization by *N. gonorrhoeae* and a model of invasive infection by *N. meningitidis.* The loop 10 hybrid antigen was as effective as full length TbpA in eliminating *N. gonorrhoeae* from the lower genital tract of female mice and was protective against the low dose invasive infection by *N. meningitidis.* These results demonstrate that TbpB or its derivatives can serve as an effective scaffold for displaying surface epitopes of integral outer membrane antigens and these antigens can elicit protection against bacterial challenge.

Keywords: protein engineering, outer membrane protein, lipoprotein, hybrid antigen, scaffold, epitope

INTRODUCTION

Neisseria gonorrhoeae, the causative agent of the sexually transmitted infection gonorrhea, relies on acquiring iron from the host iron-binding glycoproteins, transferrin (Tf) or lactoferrin (Lf), for survival in the human genitourinary tract (1, 2). This process is mediated by surface receptor proteins initially discovered in the related human pathogen *Neisseria meningitidis* (3, 4) and are presumed to be essential for survival of *N. meningitidis* both in the human upper respiratory tract during asymptomatic colonization and during invasive infection. The bacterial Tf and Lf receptor systems are each composed of an anchored lipoprotein, Tf or Lf binding protein B (TbpB, LbpB) that extends away from the bacterial surface to bind iron-loaded Tf or Lf, and the integral membrane proteins Tf or Lf binding protein A (TbpA, LbpA) that transfer iron across the outer membrane. These receptors are each exquisitely specific for human Tf and Lf (5), extending detectable binding activity only to apes but not monkeys (6). The strict specificity of TbpA for Tf was shown to be the result of mutations in sites on Tf critical for TbpA binding in response to selective pressure by the presence of the bacterial receptor proteins (7), indicating that the specificity co-evolved over 40 million years of primate divergence. It is noteworthy to recognize that the TbpA-Tf interaction is present in pathogens of multiple important food production animals, including poultry, swine, and cattle, (8, 9), suggesting that the host specificity has evolved over a period of more than 300 million years, when the Synapsids (mammalian lineage) split from Sauropsids (bird, reptile lineage).

Even prior to the experimental demonstration of the importance of the Tf receptor proteins for bacterial survival and disease causation, their presumed importance made them attractive candidates for vaccine development. The receptor complex comprised of TbpA and TbpB were shown to protect against meningococcal infection in mice (10) and shown to induce protective antibodies against *N. meningitidis* in laboratory animals (11). Although the integral outer membrane TbpA (previously denoted Tbp1) was, with notable exception (12), the essential component for *in vitro* growth (13, 14), the surface lipoprotein, TbpB (previously denoted Tbp2) became the focus for meningococcal vaccine development due to the protective immune response from purified native receptor proteins being predominantly associated with TbpB (15). The encouraging results from ongoing experiments with laboratory animals (16) led to implementing a Phase I trial in humans that was not sufficiently encouraging (17) to continue TbpB-focused meningococcal vaccine development efforts.

In order to test the potential efficacy of full-length TbpB and TbpA as vaccine antigens for protection against gonococcal infection, the intact proteins were coupled to cholera toxin B subunit (Ctb) and used for intranasal immunization of mice (18). The Ctb conjugates induced serum and vaginal antibodies and although the anti-TbpB titres were higher, the anti-TbpA antibodies were more cross-reactive. In a follow up study, regions of TbpB (N-lobe) and TbpA (loop 2) were genetically fused to the cholera A2 toxin subunit and these preparations were able to induce serum and vaginal antibodies that conferred bactericidal activity and inhibited growth dependent upon exogenous Tf (19). These results indicated that individual loop regions of TbpA were capable of inducing functional antibodies targeting intact TbpA.

Given its superior efficacy in mouse and rabbit-based studies, we hypothesized that the unexpected performance of recombinant *Neisseria* TbpB in the human Phase I trials was due to human Tf blocking important epitopes on the immunizing antigen, a limitation which would not have been present when immunizing laboratory animals due to the specificity of TbpB binding for human Tf. In an attempt to evaluate this postulate in the natural host of a Tbp-expressing pathogen, we exploited an established pig infection model. To this end, a single residue mutant of TbpB from the porcine pathogen *Haemophilus parasuis* that was defective in its binding to porcine Tf was compared to the native TbpB and a commercial vaccine product in an immunization and challenge experiment in pigs (20). The superior protection against lethal challenge provided by the non-binding *H. parasuis* TbpB protein compared to either native (wild type) TbpB or the commercial *H. parasuis* vaccine in this experiment suggests that it is worth reconsidering TbpB-based vaccines in humans. The demonstration that a Tf binding-defective *H. parasuis* TbpB is capable of inducing a cross-protective immune response against a heterologous strain expressing a TbpB variant from the same phylogenetic cluster (21) suggests that vaccine compositions with a limited number of recombinant, engineered TbpBs should be capable of inducing a comprehensive, cross-protective immune response. Taken together with the ability to produce high levels of functional and stable recombinant TbpB in the *Escherichia coli* cytoplasm, the prospects for developing TbpB-based vaccines is enticing.

Recombinant TbpA extracted and purified from the bacterial outer membrane has been shown to be effective at preventing meningococcal sepsis in a mouse infection model (22) and inducing *N. gonorrhoeae*-specific serum and vaginal antibodies in mice (18). However, there are many potential barriers to commercial production of recombinant TbpA as a vaccine antigen. Efficient production of functional, accurately folded TbpA requires insertion into the outer membrane, a process that limits production yield. The need for detergent to extract and purify TbpA and either detergents or other amphipathic reagents for maintaining stability and solubility of purified TbpA are substantial barriers for commercial development. Thus, serious consideration of TbpA as a vaccine target may require novel approaches for generating antigens capable of inducing a protective anti-TbpA response.

Protein crystallography of TbpBs and TbpB-Tf complexes (23–25) have revealed TbpB as a bi-lobed protein with individual N-terminal and C-terminal lobes each comprised of two adjacent anti-parallel β-barrel like structures with relatively unstructured loops linking the β-strands. The TbpBs thus share common features with the β-barrels of integral outer membrane proteins such as TbpA (26), however the barrel surfaces are hydrophilic such that soluble, stable recombinant proteins can be produced. We initiated a study to determine whether TbpB or its lobes could function as a scaffold to display loops from TbpA that would be capable of eliciting functional antibody against TbpA, resulting in a protective immune response. Herein, we demonstrate that these

novel antigens are able to protect against *N. gonorrhoeae* in a mouse model of mucosal colonization and against acute infection by *N. meningitidis* in a mouse model of sepsis.

MATERIALS AND METHODS

Antigen Engineering

The crystal structures of TbpA from *N. meningitidis* strain MC58 (PDB 3V8X) and TbpB from *N. meningitidis* strain M982 (PDB 3VE2) were used as a basis for antigen design. A computational model of TbpA from *N. meningitidis* strain M982 was generated using the Phyre2 server (27) with PDB 3V8X as a template. The C-lobe of the *N. meningitidis* strain M982 TbpB ($TbpB^{377-689}$) was selected as a scaffold (**Figure 1A**). The β-strands flanking the four loops on the TbpB C-lobe that were not resolved in the crystal structure (loop 20 $^{413-439}$; loop $21^{444-474}$; loop $23^{499-520}$; loop $31^{657-676}$) were selected as the sites for insertion of foreign epitopes. Loops 21, 23, and 31 are standard β-barrel loops connecting two immediately adjacent anti-parallel β-strands whereas loop 20 connects two β-strands that are quite separate as illustrated in **Figure 2** (24). The original loops were of a reasonable size (26, 30, 21, and 19 amino acids, respectively) suggesting that these locations would be amenable to hosting foreign loops of a similar size. These loops were replaced by short sections from the equivalent loops of the *Actinobacillus pleuropneumoniae* TbpB, generating the "loopless" C-lobe (LCL). The LCL was used as a scaffold to display four surface exposed regions from *N. meningitidis* strain M982 TbpA; loop 3 helix ($TbpA^{350-363}$), loop 10 ($TbpA^{815-843}$), loop 11 ($TbpA^{875-901}$) and the plug loop ($TbpA^{123-139}$). These loops were inserted into the sites of TbpB loops 20, 21, 23, and 31, respectively. Notably, loop 20 was selected for hosting the loop 3 helix region of TbpA, which is not a typical individual loop, since the anchor points were far enough apart to accommodate this region. The regions encoding foreign epitopes were introduced into the gene encoding the LCL by SOE (splicing by overlap extension)-PCR (28). The primers used for preparation of the LCL and the individual loop hybrids are listed in **Table 1**. We opted to use a scaffold lacking the N-lobe to potentially enhance the immune response against the individual loops.

To produce a hybrid antigen where the Neisseria TbpA loop 10 was hosted on a "foreign" porcine pathogen TbpB for better evaluation of protection mediated by TbpA alone, the mutant TbpB structure (PDB 4O4U) from *Haemophilus parasuis* serovar 7 was used for antigen design (**Figure 1D**). Neisseria loop 10 $TbpA^{810-840}$ was inserted into TbpB loop 31 ($TbpB^{518-522}$) of a non binding mutant of *H. parasuis* TbpB ($TbpB^{20-537}$ Y167A) by SOE-PCR and has been denoted as Hp7 + L10. The primers used for preparation of the Hp7 + L10 hybrids are listed in **Table 1**.

Engineered proteins were produced in *E. coli* strain ER2566 as described previously (20, 29). A recombinant his-tagged form of TbpA was extracted from intact cells with Elugent and purified as described previously (29) except that the Elugent was exchanged with 2% Triton X-100, and upon dilution into buffer used for immunization resulted in a final concentration less than 0.2% Triton X-100.

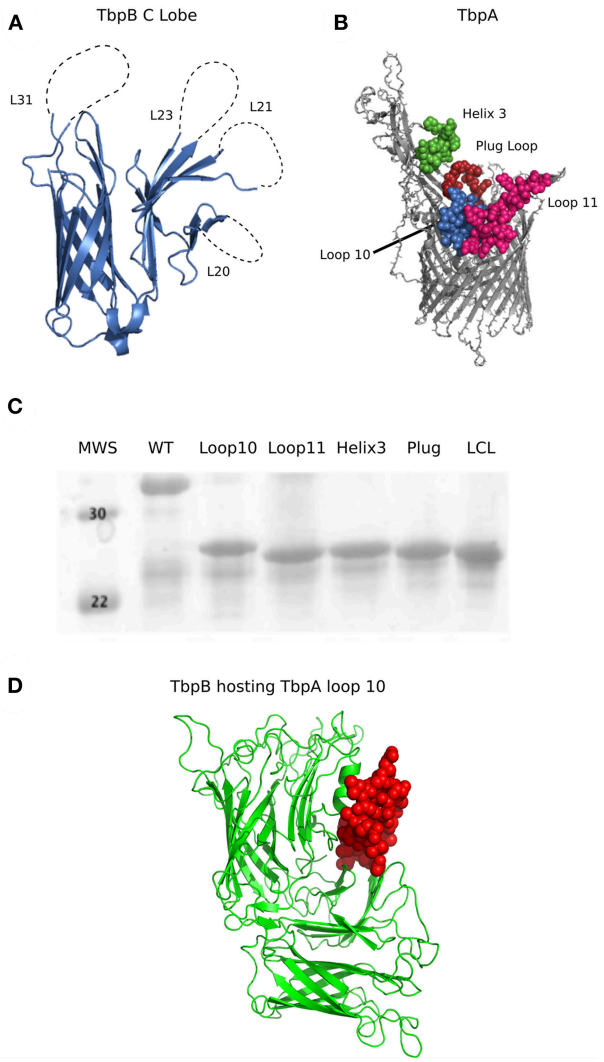

FIGURE 1 | (A) Cartoon diagram of the solved crystal structures of *N. meningitidis* TbpB C Lobe (PDB 3VE2) from strain M982. Loop 20 (26 amino acids; 413–439), loop 21 (30 amino acids; 444–474), loop 23 (21 amino acids; 499–520) and loop 31(21 amino acids; 657–676) are illustrated as dotted lines. **(B)** Surface regions of TbpA targeted for display on the hybrid antigens. Cartoon diagram of *N. meningitidis* M982 TbpA modeled with Phyre2 (27) using PDB 3V89 as a template, with the loop structures of interest shown in green (loop 3 helix), red (plug domain), pink (loop 11) and blue (loop 10). Structures on the apical side are surface facing while beta strands are within the outer membrane. **(C)** SDS-PAGE of the proteins produced from the genes encoding the wildtype C lobe, the LCL, and the four TbpA-LCL hybrid antigens that were cloned and purified. **(D)** Cartoon diagram of a Phyre2 model of a *Neisseria* TbpB (green) hosting TbpA loop 10 (red) as an example of a novel hybrid antigen displaying an exogenous loop.

Phylogenetic Evaluation

Protein sequences from Neisseria species TbpA and TbpB were collected from the Bacterial Isolate Genome Sequence Database for a total number of 4,176 TbpAs and 1,399 TbpBs. Multiple sequence alignments were done using Clustal Omega v1.2.4 (30, 31). Eight sequences were removed from the TbpA alignment that had substantial sequencing or annotation errors, leaving

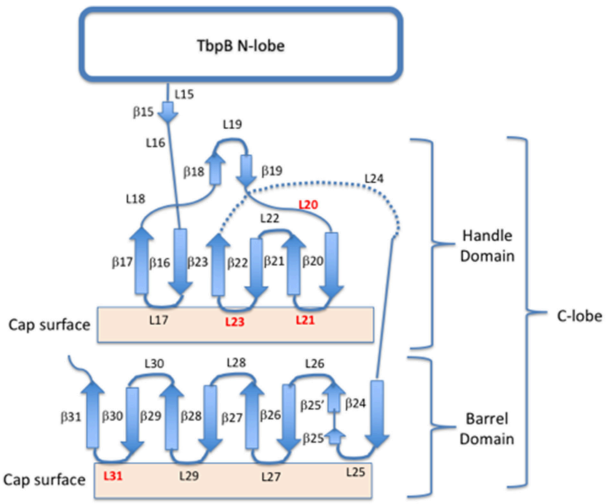

FIGURE 2 | Cartoon schematic of the insertion sites for exogenous loops on the C lobe of TbpB. Four insertion sites (loops 20, 21, 23, and 31) are shown in red, while the remaining loops and beta-sheets are annotated for both the barrel and handle domains of the C-lobe.

4,168 TbpA sequences, and 1,399 TbpB sequences. Phylogenetic trees were built using RaxML v8.2.12 with the WAG evolutionary model and a gamma distribution of rates across sites (32).

Sequences of TbpAs from a panel of *N. meningitidis* and *N. gonorrhoeae* strains were evaluated for loop variability. Clustal Omega was used to produce a multiple sequence alignment (30, 31). The alignment was compared to the solved structure of MC58 TbpA [PBD #3V8X; (26)] to determine loop junctions. Alignments were visualized with Jalview 2 (33).

Animal Immunizations for *in vitro* Characterization of Serum

Female FvB mice (Charles River, seven weeks old) and New Zealand White rabbits (Reimans Fur Ranches, 3 months old) were immunized with 25 and 50 μg of antigen, respectively. Subcutaneous immunizations were performed on days 0, 21 and 42 and were composed of 20% Emulsigen D (MVP adjuvants) in sterile PBS to a final volume of 100 μl (mice) or 500 μl (rabbits) and a final cardiac bleed was done on day 56. Animal immunizations and bleeds were performed in accordance with the University of Calgary Animal Care Committee under protocol AC11-0033.

Strain Construction

A TbpB knockout strain of *N. meningitidis* M982 was constructed by taking advantage of natural transformation. Flanking regions upstream and downstream of the *tbpB* gene were PCR amplified and a kanamycin resistance cassette was inserted between the regions. The PCR amplified DNA was mixed with a cell suspension that was applied to chocolate agar plates and incubated overnight. The cells were then re-suspended and plated onto PC55 chocolate agar (Enriched, Dalynn Biologicals) with 50 μg/ml kanamycin for selection and were confirmed by sequencing. Primers used to prepare the *tbpB* knockout strain are listed in **Table 1**.

Whole Cell ELISA

N. meningitidis and *N. gonorrhoeae* were grown overnight on chocolate agar and colonies were resuspended in BHI supplemented with 100 μmol/L deferoxamine mesylate salt ("desferal," Sigma) and grown for 6 h. Cells were resuspended in phosphate buffered saline (PBS) and heat killed at 56°C for 30 min. Cells were resuspended to an OD_{600} 0.4–0.5 in PBS and either 50 μl per well was added to 96-well plates or 20 μl per well was added to 384-well plates and dried overnight. Plates were washed with PBST and then blocked at room temperature with 5% skim milk or 5% bovine serum albumin (BSA) in PBST for 1 h. Sera were added in two-fold dilutions starting at a dilution of 1:500, followed by incubation with a secondary antibody [1:50,000 dilution, Anti-Mouse IgG (H&L) peroxidase antibody (Rockland Inc.) or 1:10,000 dilution, Goat Anti-Mouse IgG (H&L) peroxidase antibody (Jackson ImmunoResearch Laboratories Inc.)], washed and developed (tetra-methyl benzoate, TMB, Sigma). Development was quenched with 1 M H_2SO_4 and plates were read at 450 nm.

Growth Assay

Due to limited volumes of mouse sera, these experiments were performed with rabbit sera. *N. meningitidis* M982ΔtbpB was grown overnight on chocolate plates at 37°C with 5% CO_2 and then sub-cultured into BHI supplemented with 100 μmol/L desferal and grown for 30 min. 10% v/v heat-killed rabbit sera were added to each culture, grown for 2 h, followed by the addition of hTf and grown for a further 2 h. The viable cell count was determined after dilution and plating on BHI agar grown overnight. LCL sera were initially compared to naïve rabbit sera with no significant differences noted. LCL sera were then used as a control.

Serum Bactericidal Assay

A modified version of the standard bactericidal assay (34) was used to compare the different sera. *N. meningitidis* strains were grown overnight and re-streaked on chocolate agar with 100 μmol/L desferal and grown for 4 h. Two-fold dilutions of individual heat-killed mouse sera starting at a 1:4 dilution were added to Hank's Buffered Salt Solution (with $CaCl_2$ and $MgCl_2$, Gibco) and equal volumes diluted bacteria and baby rabbit complement were added (25% v/v each). Bacteria were plated on BHI agar at time zero and after a 60-min incubation at 37°C for enumeration. Bactericidal level was defined as the inverse of the last dilution of sera for which more than 50% of the bacteria were killed compared with the control.

Lower Genital Tract Infection With *N. gonorrhoeae*

Two cohorts of female 5-week old C57Bl/6 mice ($n = 9$–11 mice per immunization group, per cohort) were immunized three times intra-peritoneally (i.p.) with one of TbpA, Hp7 + L10 or adjuvant (alum; aluminum hydroxide) alone. All immunizations were composed of 25 μg of antigen in sterile PBS formulated

TABLE 1 | Oligonucleotide Primers.

No.	Name	Protein sequence	Sequence
4012	C-lobefor	GSSSENK	GCGC**GGATCC**TCGTCTGAAAACAGTAAGCTG
3181	C-loberev	KRQQPVQ*	ATGCCAGT**AAGCTT**TTATTGCACAGGCTGTTGGCGTTTC
4046	LCLlp1rev	VDGGIMIDLAG	TCCGGCCAGATCAATCATAATGCCGTCGAC
4045	LCLlp1for	ILDLAGTEFTR	ATTGATCTGGCCGGAACAGAATTTACCCGC
4048	LCLlp2rev	RKFEHTTINGK	TTTGCCGTTAATCGTGTGTTCAAATTTGCG
4047	LCLlp2for	TINGKTYEVE	ACGATTAACGGCAAAACCTATGAAGTCGAA
4050	LCLlp3rev	GMLTRKGKQV	AACCTGTTTGCCTTTGCGCGTCAACATTCC
4049	LCLlp3for	KGKQVEQSMF	AAAGGCAAACAGGTTGAACAAAGTATGTTC
4052	LCLlp4rev	GGWFAYHKSDNGS	GCTGCCGTTATCGCTTTTATGATAGGCAAACCATCCGCC
4151	LCLlp4for	YHKSDNGSATVVF	TATCATAAAAGCGATAACGGCAGCGCGACCGTGGTATTC
4157	LCLlp1-helixrev	IPLLPATKAVFDENRK	ATTTGCGGTTCTCATCAAAGACGGCTTTTGTGGCTGGGAGGAGTGGAAT
4156	LCLlp1-helixfor	AVFDENRKYGPEFTRKFE	GCCGTCTTTGATGAGAACCGCAAATACGGCCCGGAATTTACCCGCAAATTTGAA
4159	LCLlp2-lp10rev	TRKFEHTAKEITELLGSRALLNGNSRN	ATTGCGGCTGTTGCCGTTGAGCAAAGCCCGGCTGCCCAACAACTCTGTGATTTC CTTGGCCGTGTGTTCAAATTTGCGGGT
4158	LCLlp2-lp10for	SRALLNGNSRNTKATARRTRTYEVEVC	AGCCGGGCTTTGCTCAACGGCAACAGCCGCAATACAAAAGCCACCGCGCGCC GTACCCGCACCTATGAAGTCGAAGTCTGC
4161	LCLlp3-Lp11rev	YGMLTRKVTWENVRQTAGGAVNQHK	TTGTGTTGGTTGACTGCGCCGCCGGCAGTTTGCCGCACATTTTCCCAAGTAACT TTGCGCGTCAACATTCCGTA
4160	LCLlp3-Lp11for	AGGAVNQHKNVGVYNRYAVEQSMFL	GCCGGCGGCGCAGTCAACCAACACAAAAATGTCGGCGTTTACAACCGATATGC CGTTGAACAAAGTATGTTCCTC
4163	LCLlp4-pluglprev	LGGWFAYTAQAALGGTRT	CGTCCTCGTCCCGCCCAATGCCGCCTGCGCGGTATAGGCAAACCATCCGCCCAA
4162	LCLlp4-pluglpfor	AALGGTRTAGSSATVV-FG	GCGGCATTGGGCGGGACGAGGACGGCGGGCAGCAGCGCGACCGTGGTATTCGGT
4755	HpTbpBfor	GSGVSKE	TGAACA**GGATCC**GGCGTGTCTAAAGAAG
4756	HpTbpBrev	GAKQQVKK	TGTTCA**ACTAGT**TTATTTTTTTACTTGTTGTTTTGCACC
4772	RvsPos4uplp10	LGGQFRYQKAKE	CCTTGGCTTTTTGATAACGGAATTGTCCGCCAA
4773	FwdPos4uplp10	GQFRYQKAKEITELLG	GACAATTCCGTTATCAAAAAGCCAAGGAAATCACAGAGTTGTTGGG
4774	Revlp10Pos4dn	ARRTRPVGVGAVFGAKQQ	CTTGTTGTTTTGCACCAAAGACAGCACCTACACCTACAGGGCGGGTACGGCGCG
	TbpBKO_UpF		ATGACGCGATTAGAGTTTCA
	TbpBKO_UpR	MNNPLVI (TbpB)	GGATATCAGCTGGATGGCAATCACCAATGGATTGTTCATA
	TbpBKO_DownF		TAATTGGTTGTAACACTGGCAGCACGGCTGCCGAACAATC
	TbpBKO_DownR	YSIRGMD (TbpA)	CCATACCGCGTATCGAGTAG
4600	Kan_F		TTGCCATCCAGCTGATATCC
4601	Kan_R		GCCAGTGTTACAACCAATTA

Underlined residues are sequences of the inserted loops.

with 100 μg aluminum hydroxide (Sigma-Aldrich) in 100 μl total volume, and were administered on day 0, 14, and 28. Mice were treated with three doses of water-soluble 17β-estradiol (0.5 mg per mouse per dose, administered day −2, 0, and 2 relative to the day of infection) to induce susceptibility to gonococcal infection and daily antibiotics (vancomycin, streptomycin and trimethoprim, beginning at day −2) to reduce commensal bacteria in the lower genital tract, as described previously (35, 36). *N. gonorrhoeae* strain MS11 was grown on GC agar supplemented with isovitalex (Fisher Scientific) at 37°C with 5% CO_2 overnight. Mice were given approximately 10^7 CFU of *N. gonorrhoeae* resuspended in sterile PBS [with $CaCl_2$ and $MgCl_2$ (PBS++)] intra-vaginally. Starting on day 1 post infection, vaginal lavages were performed with 10 μl of sterile PBS++ and plated on GC agar supplemented with isovitalex and VCNT inhibitor to determine bacterial burden. Mice that did not have recoverable gonococci on day 1 post infection were removed from analysis, which may be due to perturbations in the vaginal microbiota as has been previously reported (37). Colonies were validated to be gonococci by colony PCR with gonococcal-specific primers. Animal work was performed in accordance with University of Toronto Animal Ethics Review Committee under protocol 20011319.

Invasive Challenge With *N. meningitidis*

Two cohorts of male C57Bl/6 mice (Charles River, 5 weeks of age) were immunized i.p. three times with one of TbpA or Hp7 + L10 formulated with 100 μg aluminum hydroxide (Sigma-Aldrich), or alum alone ($n = 4$–6 per immunization group, per cohort). Vaccines were formulated with 25 μg of antigen in sterile PBS in a total volume of 100 μl per mouse, and given on day 0, 14, and 28. Sepsis modeling was performed as described previously (38). Briefly, mice were challenged with either 2.5×10^7 CFU (cohort 1) or 7.5×10^7 CFU (cohort 2) of *N. meningitidis*

strain M982. *N. meningitidis* was grown overnight on GC agar supplemented with isovitalex (Fisher Scientific) at 37°C with 5% CO_2. On the day of infection, *N. meningitidis* was resuspended at an OD_{600} of 0.1 in RPMI broth and grown for 4 h with shaking at 37°C. Mice were infected via i.p. injection under isofluorane anesthesia. At the time of infection, mice were also given a separate i.p. injection on the contralateral side containing 8 mg of iron-loaded human transferrin (Sigma-Aldrich) resuspended in sterile PBS. Mice were monitored at 3, 12, 18, 24, 36, and 48 h post infection and tail bleeds were collected at 3, 18, 36, and 48 h post infection or at any time clinical endpoint was reached. Tail bleeds were plated on Difco GC agar (BD Biosciences) supplemented with isovitalex and antibiotic selection (BD BBL VCNT Inhibitor, Fisher Scientific) to determine bacterial burden in the blood. Animal work was performed in accordance with University of Toronto Animal Ethics Review Committee under protocol 20011775.

Statistical Analyses

Statistical analysis and figures were completed with GraphPad Prism (version 7.0 for Mac OS X; GraphPad, San Diego, CA). Whole-cell ELISA titres were compared with either an ordinary one-way ANOVA with *post-hoc* comparisions performed with Dunnett's multiple comparisons test (**Figures 6A,B**) or a two-way ANOVA with *post-hoc* comparisons performed with Sidak's multiple comparisons test (**Figure 5A**). The ability of sera to block transferrin-based growth was compared with an ordinary one-way ANOVA with *post-hoc* comparisons performed by the Holm-Sidak test (**Figure 5C**). Pre-challenge rabbit serum was compared to LCL immune serum by an unpaired t-test (**Figure 5B**). The duration of gonococcal colonization was evaluated by log rank [Mantel-Cox] test (**Figure 7**).

Transfer of Methodology

All protocols, methodology and study materials will be made available upon request.

RESULTS

Design and Production of Hybrid Antigens

The lobes of the TbpB and LbpB lipoproteins are comprised of two adjacent anti-parallel β-barrel like structures. The loops linking the β-strands can be quite large (> 80 amino acids), particularly the negatively charged regions in the LbpB C-lobe (39), suggesting that the C-lobe can accommodate large loop insertions. Since the loops in the N-lobe region comprise the binding interface with Tf, we considered that targeting the loops in the N-lobe for replacement could interfere with generation of antibodies that could block binding and use of Tf. Therefore, the C-lobe was selected as the preferred scaffold for heterologous epitope display. When designing a scaffold derived from the TbpB of *Neisseria,* we selected as insertion sites the four loops in the C-lobe that were not resolved in the crystal structure of a meningococcal TbpB (25), as these were relatively large and flexible (26, 30, 21, and 19 amino acids, respectively). A preliminary scaffold was prepared by replacing the existing loops on the *N. meningitidis* strain M982 TbpB C-lobe (**Figure 1A**) with small segments from loop regions of TbpB from *A. pleuropneumoniae* (23).

To test the versatility of the scaffold protein to host exogenous epitopes, we selected four different peptide segments of varying size from surface-exposed regions of the *N. meningitidis* strain M982 TbpA to display. Using the structure of meningococcal TbpA in complex with human Tf as a guide (26), we selected three regions from the extracellular loops extending off the beta-barrel and one from the plug region (**Figure 1B**) as these regions appeared to be surface exposed and potentially important for TbpA functionality. The two largest inserts consisted of the majority of the sequence of external loops 10 (30 residues) and 11 (27 residues), best representing the effective transplant of substantial segments of external loop regions. The TbpA loop 3 alpha-helix was inserted into the TbpB loop 20 site in which the anchoring β-strands are not adjacent so that the alpha-helical region could be accommodated. Insertion of these individual regions (**Figure 1C**) or a combination of the four loop insertions

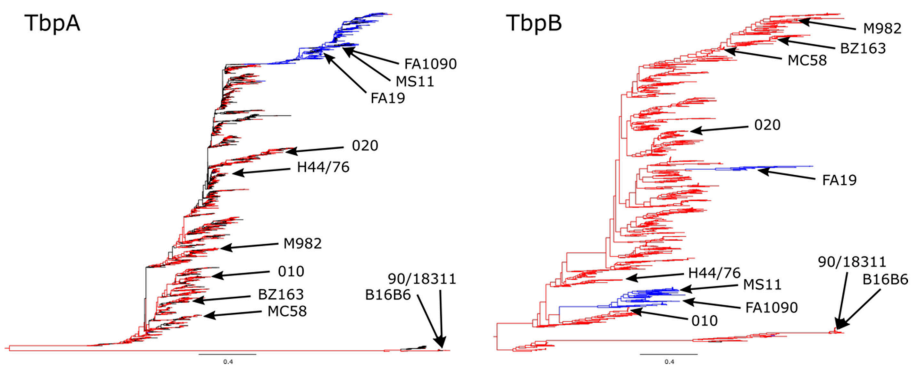

FIGURE 3 | Phylogenetic analysis of Neisseria species TbpA **(Left)** and TbpB **(Right)** protein sequences. *N. meningitidis* sequences are shown in red, *N. gonorrhoeae* sequences are shown in blue, and any commensal *Nesseria* sequences or sequences lacking species annotation are shown in black, with all of the variants described in the (**Figure 4**) alignment denoted on the trees. The trees are shown at the same scale (0.4) in order to better compare the extent of diversity between the proteins. *N. meningitidis* isotype I TbpBs (including known isotype I containing strains B16B6 and 90/18311) are shown at the bottom with the corresponding TbpAs also shown at the bottom of the trees.

(data not shown) did not have a substantial impact on the production or stability of the resulting recombinant proteins.

Phylogenetic analysis of TbpA and TbpB sequences from *N. meningitidis* and *N. gonorrhoeae* have demonstrated that the gonococcal diversity exists predominantly as either one subset (TbpA) or two subsets (TbpB) of the isotype II-type meningococcal diversity (**Figure 3**). When compared at the same scale, TbpB contains substantially more variability than does TbpA within each isotype, however the isotype distinction appears in both TbpA and TbpB sequences, with strains appearing to be consistently isotype I or isotype II, as previously reported (40).

To evaluate the potential of hybrid antigens to induce a cross-reactive antibody response, we examined the sequence diversity of the selected loops. The sequences of the chosen TbpA loops were compared across a collection of three *N. gonorrhoeae* strains (**Figure 4**, bottom three sequences) and eight *N. meningitidis* strains (**Figure 4**, top eight sequences) selected to represent the overall diversity of *Neisseria* TbpAs (as seen in **Figure 3**). The overall sequence diversity of TbpA parallels the sequence diversity of the TbpB C-lobe, which divides the C-lobe variants into an isotype I cluster with a smaller size C-lobe (also reflected in the overall TbpB size) and a larger size isotype II C-lobe (40). Thus, the *N. meningitidis* collection included six strains expressing isotype II TbpBs and two strains expressing isotype I TbpBs (B16B6 and 90/18311) while all three *N. gonorrhoeae* strains expressed isotype II TbpBs. There were two variants within loop 11 (**Figure 4B**) and three variants of loop 10 (**Figure 4A**), illustrating that these are two of the most conserved surface loops on TbpA. The loop 3 helix region (10 residues) and the plug domain (15 residues) were selected due to the critical function they are proposed to serve in removing iron from Tf (26). In the TbpA-hTf complex, the loop 3 helix is inserted between the C1 and C2 subdomains of transferrin while the plug loop interacts directly with the C1 subdomain, together coordinating the release of iron (26). The plug domain is highly conserved among the representative TbpAs, with only *N. gonorrhoeae* strain FA1090 differing by a single residue (**Figure 4D**). There is more variability present in the loop 3 helix (**Figure 4C**), with substantive differences between variants from meningococcal strains expressing isotype I and II TbpBs as well as additional variability within the *N. gonorrhoeae* variants. Each of these four components was spliced into the LCL backbone and each hybrid antigen was expressed, purified (**Figure 1C**) and used for initial immunizations in mice and rabbits to collect sera for *in vitro* evaluation.

Ability of Hybrid Antigens to Elicit Functional Antibodies

In order to evaluate whether sera raised against the hybrid antigens recognized native epitopes on TbpA at the surface of the bacterial cell, a whole cell ELISA was performed with wild type *N. meningitidis* strain M982 (the homologous strain) and with a TbpB deletion mutant (M982ΔTbpB) to eliminate reactivity due

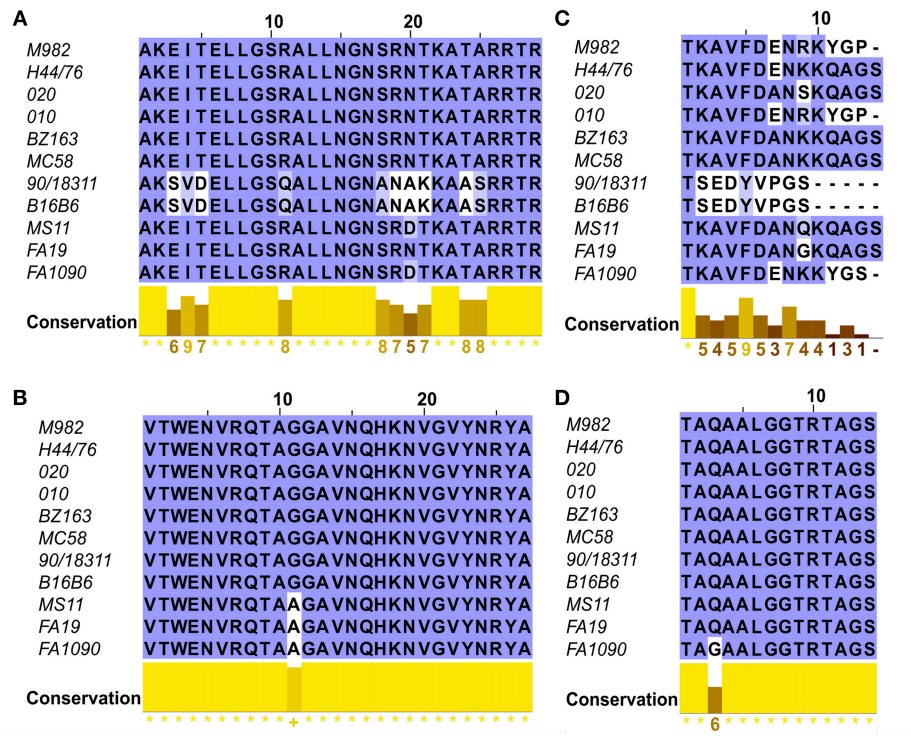

FIGURE 4 | Sequence alignments of the loops of TbpA used in the hybrid antigens across 11 strains including six *N. meningitidis* isotype 2 strains (M982, H44/76, 020, 010, BZ163 and MC58), two *N. meningitidis* isotype 1 strains (B16B6 and 90/18311) and three *N. gonorrhoeae* strains (MS11, FA19 and FA1090). Clustal Omega amino acid alignments displayed in JalView of the four regions of TbpA of interest include **(A)** Loop 10; **(B)** Loop 11; **(C)** Helix 3 and **(D)** the Plug Domain.

to antibodies against the TbpB-derived scaffold. Sera against the hybrid antigens had higher titres against wild-type M982 (black bars, **Figure 5A**) than the sera against TbpA, likely reflecting the greater accessibility of TbpB at the cell surface. Notably, anti-loop 10 hybrid titres against M982ΔTbpB (gray bars, **Figure 5A**) were comparable to the anti-TbpA titres, suggesting that loop 10 is accessible to antibody and that the hybrid antigen efficiently induced antibodies that recognize the native antigen. Antisera raised against loop 11 also displayed significant reactivity against TbpA compared to serum against adjuvant alone. Comparatively low reactivity against M982ΔTbpB was observed with antisera derived from the hybrid loop 3 helix or plug loop immunizations.

To determine if the TbpA loop-specific antibodies raised upon immunization with the hybrid antigens are able to functionally block Tf binding and therefore inhibit growth, the M982ΔTbpB strain was grown with Tf as the sole iron source in the presence of heat-inactivated immune rabbit sera (**Figure 5C**). We opted to use a strain lacking TbpB to ensure that only antibodies against

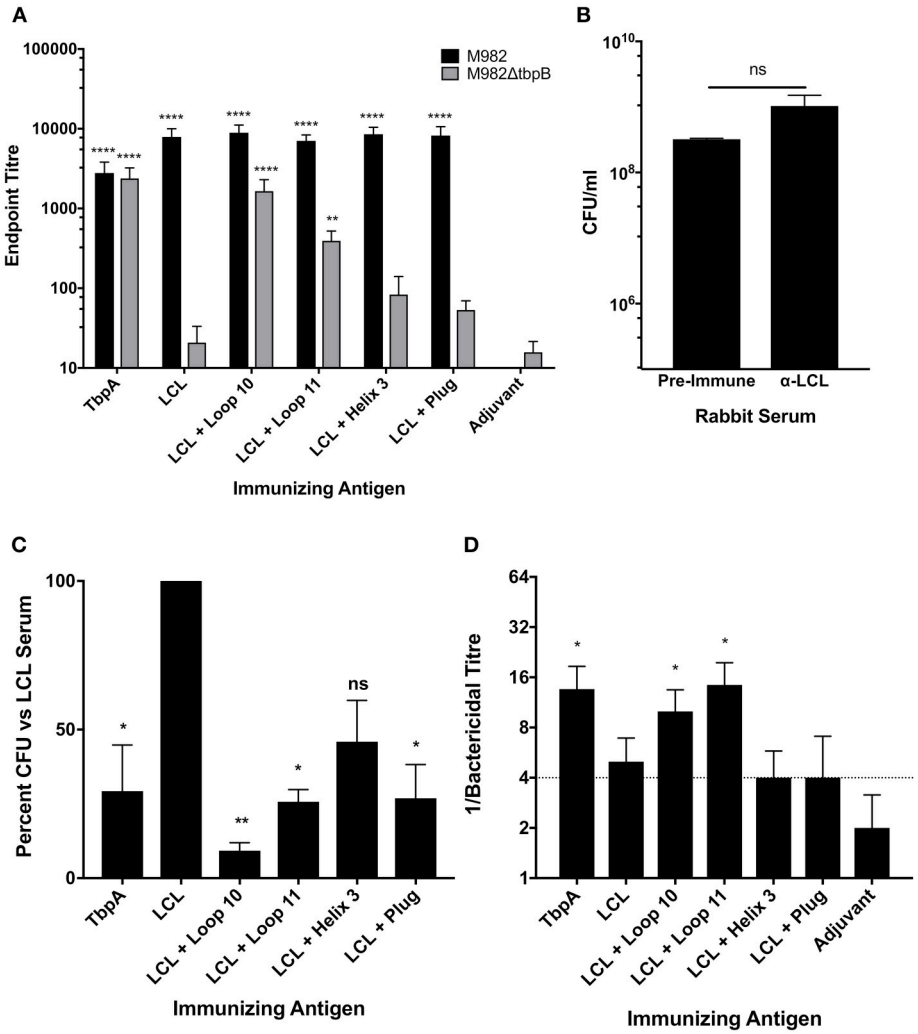

FIGURE 5 | *In vitro* characterization of the LCL scaffold and the LCL-TbpA hybrid antigens. **(A)** Mouse sera were evaluated in a whole cell ELISA against both wild-type *N. meningitidis* M982 (Black) and M982 with TbpB knocked out (M982ΔtbpB; Gray). Sera were assayed in triplicate from individual mice (4 to 5 mice per group) and averaged and displayed as mean +/- SEM. Significance was determined as a significant increase in titer compared with mice that received adjuvant alone by two-way ANOVA with Sidak's multiple comparison test for both wildtype and knockout data sets. $^{**}p \leq 0.01$, $^{****}p \leq 0.0001$. **(B,C)** Iron starved *N. meningitidis* M982ΔtbpB was grown with antisera from rabbits immunized with the antigens of interest. Iron-loaded hTf was added and bacteria were grown for 2.5 h and enumerated. Bacteria supplemented with LCL antiserum grew at the same rate as those supplemented with pre-challenge rabbit sera **(B)**, as compared by an unpaired *t*-test. Growth was significantly decreased in bacteria supplemented with whole TbpA antisera, as well as antisera from rabbits immunized with LCL + Loop 10, Loop 11 and the plug domain **(C)**. Graph represents data generated from three independent experiments plated in duplicate. Significance was determined as a significant decrease in final CFU/ml compared with bacteria grown with LCL alone by ordinary one-way ANOVA with *post-hoc* analysis by Holm-Sidak's multiple comparison test. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$. **(D)** Serum bactericidal activity of mouse sera. Sera from each mouse was assayed against *N. meningitidis* strain M982 lacking TbpB. Data shown are averages of 4 to 5 mice per group ± SEM. A bactericidal titer of ¼ is the limit of detection of bacterial killing. Some values fall below the cut off due to some animals within the group showing killing at the 1:4 dilution while others produced no bacterial killing.

TbpA were responsible for any observed inhibition of growth. Growth in the presence of antiserum raised against the TbpB-derived LCL was not statistically different from growth in the presence of pre-challenge (naïve) rabbit serum (**Figure 5B**) and represents baseline, while the 70.7% reduction in CFU with the anti-TbpA antiserum demonstrates that growth inhibition can be achieved. Antisera raised against the loop 11 and plug loop hybrid proteins showed levels of inhibition (74.3 and 73.1%, respectively) similar to that obtained with anti-TbpA; this is particularly notable when considering that the titer of plug-specific antibodies was low, suggesting that antibodies which target it are highly effective at blocking Tf-dependent growth. However, the loop 10 hybrid elicited the strongest growth inhibition, with a reduction in CFU of over 90% compared to bacteria grown in the presence of LCL.

Serum bactericidal assays were performed to quantify complement-dependent killing activity of mouse sera (**Figure 5D**) against the M982ΔTbpB strain, reflecting bactericidal activity due to anti-TbpA antibodies alone. Using sera from mice immunized with either the loop 10 or loop 11 hybrid proteins, bactericidal activity against the M982ΔTbpB strain occurred in dilutions above 1:8, comparable to the anti-TbpA antisera, indicating that antibodies raised to TbpA loops 10 and 11 alone are sufficient to produce bacterial killing *in vitro*.

Ability of the Loop 10 Hybrid Antigen to Induce Protection Against Colonization and Sepsis

Since the loop 10 hybrid antigen elicited sera that showed the best reactivity to whole bacterial cells, was bactericidal against TbpA, and was the best at blocking Tf-dependent growth *in vitro*, this antigen was prioritized for evaluation in two separate protection studies. The TbpA loop 10 differs by only one residue between *N. gonorrhoeae* strain MS11 and *N. meningitidis* strain M982 (**Figure 4A**), suggesting that the same hybrid antigen could be evaluated against both lower genital tract infection by *N. gonorrhoeae* and invasive challenge by *N. meningitidis*. The TbpA loop 10 from *N. meningitidis* strain M982 was transplanted onto a foreign TbpB from the porcine pathogen *Haemophilus parasuis* (**Figure 1D**) to avoid the immune response against TbpB induced by the LCL obscuring protection mediated by the TbpA loop 10 when challenging with wild type bacteria (expressing *Neisseria* TbpB).

To test for protection against *N. gonorrhoeae* infection in the female lower genital tract, recombinant TbpA from *N. meningitidis* strain M982 or the *H. parasuis* TbpB hosting the *Neisseria* loop 10 were used to immunize wild type, female C57Bl/6 mice (**Figure 7**). *H. parasuis* TbpB hosting the *Neisseria* loop 10 was highly immunogenic in mice as shown by serum IgG levels against heat-killed *N. gonorrhoeae* (**Figure 6A**) and *N. meningitidis* (**Figure 6B**) in a whole-cell ELISA, while mice immunized with TbpA showed an increase in reactivity against whole bacteria that did not reach statistical significance compared to mice immunized with alum alone. The immunized female C57Bl/6 mice were challenged intra-vaginally with 10^7 CFU of *N. gonorrhoeae* strain MS11. Mice that received alum were robustly colonized with a median colonization period of 10 days between the two cohorts (**Figures 7A,B**). In contrast, mice immunized with *Neisseria* TbpA had a median colonization duration of 6 days, while mice immunized with the *H. parasuis* TbpB hosting loop 10 had a median colonization duration of only 4 days.

To measure protection against invasive meningococcal disease, male C57Bl/6 mice were immunized with the two antigens or alum, and then challenged parenterally with *N. meningitidis* strain M982, the homologous strain to the antigens in use (**Figure 8**). In this experiment mice were infected in two cohorts with either a low (2.5×10^7 CFU per mouse) or high (7.5×10^7 CFU per mouse) dose of *N. meningitidis*. The hybrid antigen displaying loop 10 conferred protection superior to that with TbpA upon low dose challenge; all mice immunized with the hybrid antigen survived the low dose challenge, had relatively low clinical scores, and there were no bacteria detected in blood after 24 h (**Figure 8A**). In contrast, the mice immunized with this hybrid antigen challenged at the high dose had a similar outcome to the adjuvant treated mice (**Figure 8B**). In future experiments, it will be important to determine whether using a combination of hybrid antigens displaying different loops at different sites on TbpB can overcome the lack of protection observed when the immune response is directed against a single hybrid antigen.

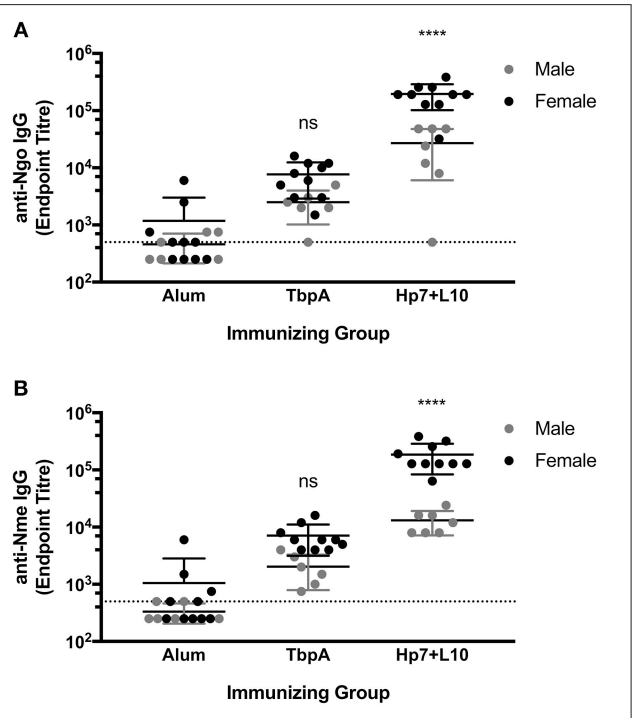

FIGURE 6 | Whole-cell serum IgG ELISA titres of mice immunized with alum, TbpA or Hp7-L10 when captured by **(A)** heat-killed *N. gonorrhoeae* strain MS11 or **(B)** heat-killed *N. meningitidis* strain M982. Female mice were used in the lower genital tract gonococcal colonization, while male mice were challenged systemically with *N. meningitidis*. Significance was determined as a significant increase in titer compared with mice that received alum alone by ordinary one-way ANOVA with *post-hoc* analysis by Dunnett's multiple comparisons test. $^{****}p < 0.0001$.

The results with mice immunized with the native TbpA was surprising in that there was little protection observed at the low challenge dose (Panel A) and a modest level of protection observed at the high challenge dose (Panel B). Since the TbpA used in the low dose and high dose experiments were from different preparations, we cannot exclude the possibility that the low and variable level of protection observed could be due to differences in the conformation and state of the protein used for immunization or, alternatively, due to formulation effects such as its association with the adjuvant. It is noteworthy that early experiments suggested that TbpA would not be protective (11, 15), likely due to inconsistencies in the conformation and stability of the protein. Subsequent studies demonstrated effective protection, and heterologous strain cross-protection, against challenge in the sepsis model (22). Collectively, these results highlight the challenges that could be encountered for commercial production of a TbpA antigen.

DISCUSSION

The pursuit of a vaccine for prevention of gonorrhea has been a long-standing goal, with early efforts at developing a pilus-based vaccine culminating in disappointing results in a clinical trial (41). In spite of some pessimism at the time regarding the feasibility of developing a gonococcal vaccine, continued efforts by multiple groups have led to a number of promising targets in the intervening years (42). The growing prevalence of strains resistant to multiple antibiotics is increasing the potential for non-treatable cases of gonorrhea (43), providing a renewed sense of urgency for the development of an effective vaccine for disease prevention.

The development and implementation of an experimental gonococcal infection model in male volunteers (44) has been an invaluable tool for providing insights into the interaction between gonococci and the host, and in evaluating potential vaccine targets. The ability of gonococcal strains deficient in IgA protease, pili (PilE), or Opa proteins to remain infectious in this model suggests that these proteins are not essential and thus may not be ideal vaccine targets, although one cannot exclude the possibility that they mediate critical steps in the natural infectious process. In contrast, a strain deficient in the transferrin receptor was unable to persist (1), which at the time was unexpected since the concept of transferrin being inside the body and lactoferrin being present on the mucosal surface was the prevailing view of iron homeostasis. Since this experiment was performed with a gonococcal strain naturally lacking a functional lactoferrin receptor, a follow up study was initiated to compare the roles of these two iron acquisition systems (2). Restoration of a functional lactoferrin receptor in the strain lacking the transferrin receptor enabled it to establish infection in a substantial proportion of the volunteers, demonstrating that either receptor could support growth on the mucosal surface of the male genitourinary tract.

A competition study with strains expressing a functional Tf receptor and either possessing or lacking a functional Lf receptor demonstrated that all of the persisting bacteria had both receptors (2). Although it was not possible to establish a mechanism for this competitive advantage, Lf binding protein B (LbpB)-mediated protection against cationic peptides may have contributed to this advantage (39). Indirect assessment of mucosal concentrations of Tf and Lf by measuring their concentrations in urine indicated that, prior to infectious challenge, the median concentration of Tf was 2.1-fold higher than Lf (2). This indicates that Tf is present on the mucosal surface in the absence of significant

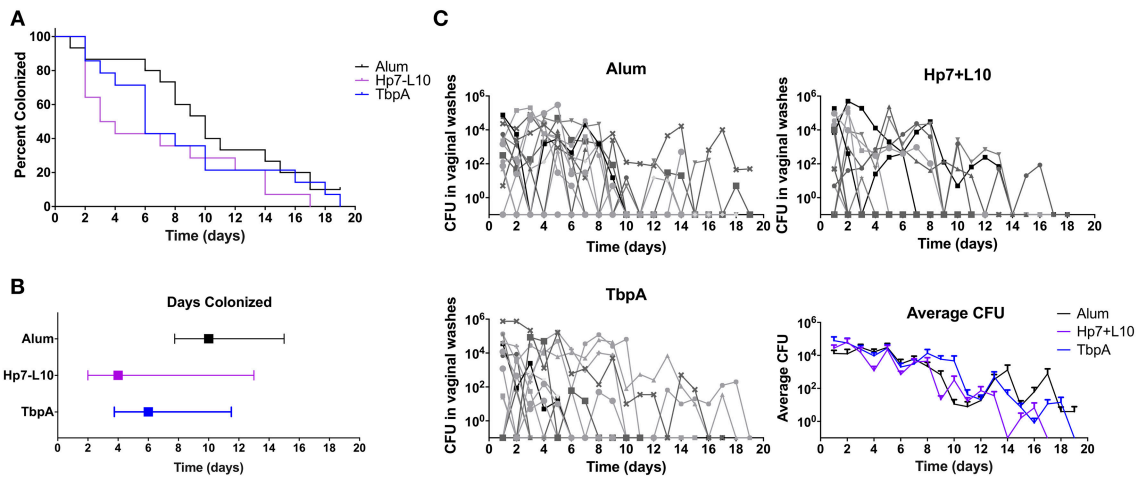

FIGURE 7 | Lower genital tract gonococcal colonization in immunized female mice. Mice were immunized with one of TbpA (17 mice, with 2 excluded due to non-infection; blue), *H. parasuis* TbpB hosting the meningococcal TbpA loop 10 (17 mice, with 5 excluded due to non-infection; purple) or alum alone (14 mice, with 5 excluded due to non-infection; black) and challenged with 10^7 CFU of *N. gonorrhoeae* intra-vaginally. Mice were immunized and challenged in two cohorts and are shown as combined data. Duration of colonization is shown as percent **(A)** as well as by median with interquartile range **(B)**. The recovered CFU from vaginal washes from each mouse is shown **(C)** broken down by each immunization group as well as averages are shown. Duration of gonococcal colonization was compared by a log rank [Mantel-Cox] test, however no significance was observed.

inflammation, however the mechanism for delivery of Tf to the mucosal surface remains unknown. Several days after challenge, the levels of Lf, but not of Tf, rose. The rise in Lf is consistent with its release by degranulating neutrophils at the site of infection, and the lack of increase of Tf suggests that the delivery of Tf to the mucosal surface is not due to leakage during inflammation.

Although we do not have any information regarding the specific role of each component of the Tf receptor from the human male urethral infection model, studies with the pig pathogen *Actinobacillus pleuropneumoniae* indicate that TbpB is essential for persistence and infection in the porcine respiratory tract (45). The invariant presence of TbpB in *N. gonorrhoeae, N. meningitidis* and all other human and animal pathogens that possess the bipartite TbpB-TbpA receptor studied to date make clear that TbpB is essential for the efficient capture of iron-containing Tf on the mucosal surface. Recent studies with a second pig pathogen, *H. parasuis,* demonstrated that a Tf binding-defective mutant of TbpB elicited dramatically superior protection relative to either native TbpB or a commercially-available vaccine in an invasive infection model (20). These results provide an explanation for the disappointing results from a Phase I trial in humans (17), which used a binding-competent TbpB from *N. meningitidis*; a binding-defective mutant would presumably also be superior in humans. Through the bioinformatic analysis of TbpB diversity from three porcine pathogens, immunologic analysis of the cross-reactivity of the humoral response, and additional immunization and challenge experiments (21, 46), the prospect of developing a porcine TbpB-based vaccine with two or three mutant TbpBs for prevention of infection by three pathogens seems feasible. In addition, recent preliminary results suggest that immunization of piglets in a production facility with TbpB-based vaccine formulations can result in eliminating natural colonization. Such sterilizing immunity is a prerequisite for any gonococcal vaccine since a vaccine that elicits asymptomatic carriage would only facilitate transmission of this already pervasive pathogen.

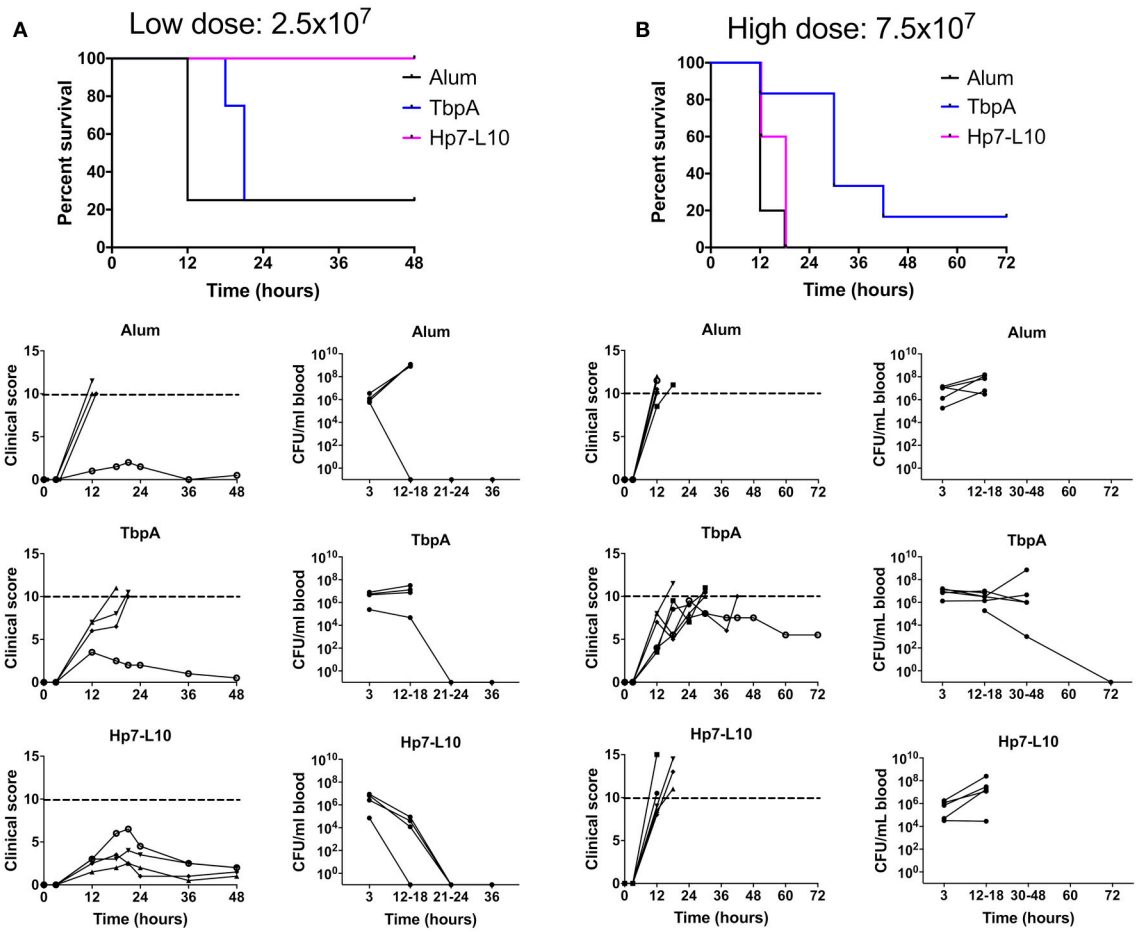

FIGURE 8 | Survival of immunized mice in a model of invasive meningococcal infection of mice immunized with either the low dose [**(A)**; 2.5 × 10^7 CFU per mouse] or the high dose [**(B)**; 7.5 × 10^7 CFU/mouse]. Mice were immunized with one of TbpA (blue), *H. parasuis* TbpB hosting the meningococcal TbpA loop 10 (Hp7-L10; purple) or alum alone (black) and challenged with a lethal dose of *N. meningitidis* strain M982 systemically. Mouse survival is shown at the top, with clinical scores and bacterial burden in the blood shown for individual mice for each immunization group (descending from the top): alum, TbpA, and *H. parasuis* TbpB hosting loop 10.

Analysis of the diversity of TbpB from strains of *N. gonorrhoeae* and other *Neisseria* species (**Figure 3**) suggests that a TbpB-based vaccine derived from two or three representative TbpBs could induce a protective response against all gonococcal isolates. However, since the gonococcus can be isolated from the upper respiratory tract and strains of *N. meningitidis* have been isolated from cases of urethritis (47), there may be ample opportunity for horizontal genetic exchange in response to strong selective pressures. Thus, the long-term efficacy of a Tf receptor-based vaccine may ultimately need to consider the reservoir of *tbpA* and *tbpB* genes in other *Neisseria* species (**Figure 3**), requiring a larger number of representative variants for a pan-*Neisseria* vaccine.

The more limited variation in TbpA sequences and its critical role in the essential iron acquisition process make it an attractive target for a gonococcal vaccine. Our *N. gonorrhoeae* colonization studies in a female mouse model showed a reduction in colonization due to immunization with TbpA (**Figure 7**) or a foreign TbpB displaying the *Neisseria* TbpA loop 10, suggesting that our hybrid antigen strategy has potential to elicit protection at the mucosal surface. It is noteworthy to mention that the *N. gonorrhoeae* challenge strain in the mouse model used in this study is not dependent upon its Tf receptor for growth in this model since only mouse (and not human) Tf is present. Thus, based on what has been observed in human studies (1), in which sustained growth and infection requires acquisition of iron from host proteins, ongoing bacterial replication in the mouse model is likely dependent upon the substantial iron stores from growth of the large inoculums on iron rich media prior to challenge. This clearly contrasts the more physiologically-relevant situation in the human male urethral model for *N. gonorrhoeae* or in the upper respiratory tract of pigs for *H. parasuis,* where bacterial survival and replication is Tf-dependent; in these cases, the anti-Tf receptor antibodies might also play a role in inhibiting the growth of the bacteria. Therefore, subsequent studies are needed utilizing transgenic mice expressing human Tf in place of mouse Tf, where the inoculum may contain bacteria with depleted levels of stored iron (achieved via growth of the bacteria in the presence of an iron chelator prior to challenge). In this instance, ongoing infection will require iron acquisition, which should reveal an even greater impact of Tbp-specific antibodies.

One other aspect that remains to be assessed in future studies is the effect of varying adjuvants. The use of aluminum hydroxide as the vaccine adjuvant in these challenge studies (chosen since it is licensed for humans) may not be optimal for inducing a protective response, and other adjuvants may provide more rapid bacterial clearance. Indeed, recent work by Russell and colleagues demonstrates that cytokine-induced polarization of the immune response can facilitate protection against *N. gonorrhoeae* infection (48, 49). Novel mucosal adjuvants are being developed in order to better target immune responses to the site of infection (50), which may be able to elicit a more robust anti-gonococcal response. Additionally, aluminum hydroxide is known to skew the immune response in a T_H2 dependent manner (51) that results in high levels of IgG1 and limited production of IgG isotypes such as mouse IgG2a or IgG2c. IgG1 has been shown to inhibit the binding of other mouse IgG isotypes to the complement factor C1q, the molecule that initiates the classical complement pathway, and thus limits the induction of IgG2a-mediated complement activation (52). This may explain why the loop 10 hybrid antigen was protective against a low but not a high dose of *N. meningitidis* in the invasive challenge model (**Figure 8**), where at a higher dose a partial inhibition of complement activation was sufficient to limit protection.

The ability of a soluble TbpB-based scaffold antigen to support exogenous loops from integral membrane proteins provides the potential to broaden the prospective breadth of antigens that can be targeted with a subunit vaccine. As the Lf receptor was able to rescue gonococcal survival in the human male urethra in the absence of the Tf receptor, expanding the scope of these antigens to contain surface exposed loops of Lf binding protein A (LbpA) may provide even broader protection while limiting the potential for vaccine escape by gonococcal isolates that express the Lf receptor.

This study demonstrated that an engineered TbpB antigen is able to display epitopes from TbpA and provide stable, immunogenic antigens that can produce anti-TbpA antibodies that are bactericidal and able to inhibit Tf-dependent growth. The proof of concept provided by this study suggests that a more comprehensive analysis of different surface loops, and of combinations of loops, will allow for an optimized immune response against TbpA. It will be important to independently assess whether the display of TbpA epitopes interferes with the cross-reactive anti-TbpB response in order to make sure the cross-protective properties of the mutant TbpBs are not compromised by the display of heterologous loops; this could presumably be overcome by including a mixture of two or more antigens with insertions at different sites. In summary, this strategy establishes the utility of our novel method of increasing the breadth of vaccine targets against diverse naturally occurring receptor variants by specifically targeting areas of interest on membrane proteins and provides the exciting potential for a single Tbp-based vaccine protective against both meningococcal and gonococcal infection.

AUTHOR CONTRIBUTIONS

AS conceived the original idea and oversaw all aspects of the study. AS consulted with TM, CC, and SC on the design of the hybrid antigens, oversaw the preparation and production of hybrid antigens by RY, and provided advice and guidance to JF in the immunization and evaluation of the immune response. TM provided oversight in the design of hybrid antigens by CC. CC examined the structures and designed the epitope transfers with input from AS and TM. RY and SC prepared the hybrid antigen genes, expressed and purified the recombinant hybrid antigens. JF performed all the functional studies with the sera including recognition of TbpA in intact cells, inhibition of growth and bactericidal activity. JF, EI, and SA designed and performed the mouse protection studies under the guidance of SG-O. JF prepared the initial draft of the manuscript and JF and AS finalized the manuscript. JF and EI prepared all figures in the manuscript.

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The *Neisseria gonorrhoeae* Methionine Sulfoxide Reductase (MsrA/B) Is a Surface Exposed, Immunogenic, Vaccine Candidate

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Control of the sexually transmitted infection gonorrhea is a major public health challenge, due to the recent emergence of multidrug resistant strains of *Neisseria gonorrhoeae*, and there is an urgent need for novel therapies or a vaccine to prevent gonococcal disease. In this study, we evaluated the methionine sulfoxide reductase (MsrA/B) of *N. gonorrhoeae* as a potential vaccine candidate, in terms of its expression, sequence conservation, localization, immunogenicity, and the functional activity of antibodies raised to it. Gonococcal MsrA/B has previously been shown to reduce methionine sulfoxide [Met(O)] to methionine (Met) in oxidized proteins and protect against oxidative stress. Here we have shown that the gene encoding MsrA/B is present, highly conserved, and expressed in all *N. gonorrhoeae* strains investigated, and we determined that MsrA/B is surface is exposed on *N. gonorrhoeae*. Recombinant MsrA/B is immunogenic, and mice immunized with MsrA/B and either aluminum hydroxide gel adjuvant or Freund's adjuvant generated a humoral immune response, with predominantly IgG1 antibodies. Higher titers of IgG2a, IgG2b, and IgG3 were detected in mice immunized with MsrA/B-Freund's adjuvant compared to MsrA/B-aluminum hydroxide adjuvant, while IgM titers were similar for both adjuvants. Antibodies generated by MsrA/B-Freund's in mice mediated bacterial killing via both serum bactericidal activity and opsonophagocytic activity. Anti-MsrA/B was also able to functionally block the activity of MsrA/B by inhibiting binding to its substrate, Met(O). We propose that recombinant MsrA/B is a promising vaccine antigen for *N. gonorrhoeae*.

Keywords: methionine sulfoxide reductase, bactericidal activity, opsonophagocytic activity, *Neisseria gonorrhoeae*, gonococcus, vaccine

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INTRODUCTION

Neisseria gonorrhoeae is an obligate human pathogen that infects human mucosal surfaces and causes the sexually transmitted infection gonorrhea. It is estimated that there are more than 106 million cases of gonorrhea worldwide each year (1). Symptomatic gonococcal infection typically presents as urethritis in males and cervicitis in females, although infection of the rectum, pharynx, and eye also occur in both sexes (2). Furthermore, asymptomatic infections are common and can occur in up to 80% of infected females and 40% of infected males. If left untreated, gonorrhea can

lead to severe sequelae, such as pelvic inflammatory disease, adverse pregnancy outcomes, neonatal complications, and infertility, and can also increase the risk of acquiring and transmitting HIV [reviewed in Edwards et al. (3)].

The recent emergence of multidrug resistant strains of *N. gonorrhoeae* has generated a major public health challenge. Cephalosporins are now the last line of defense for treating gonorrhea, however, isolates with high-level resistance to the expanded-spectrum cephalosporins, ceftriaxone and cefixime, have been identified globally (4), highlighting the requirement for novel therapeutic approaches or for a vaccine. Various potential vaccine targets have been described, however there are several challenges to developing a gonococcal vaccine. The challenges include the lack of protective immunity following infection, and the absence of a correlates of protection, as well as the high level of phase and antigenic variation of *N. gonorrhoeae* surface antigens [reviewed in Edwards et al. (3) and Rice et al. (5)]. Ideally, vaccine antigens should be conserved, immunogenic, and be able to induce functional antibodies that are able to mediate bactericidal or opsonophagocytic killing, and/or that are able to block an important function of *N. gonorrhoeae* [reviewed in Edwards et al. (3)].

Mechanisms for coping with oxidative stress are crucial for the survival of human pathogens such as *N. gonorrhoeae,* which are routinely exposed to oxidative killing by the host and are frequently isolated within polymorphonuclear leukocytes (PMNs) (6). Methionine residues in proteins can easily be oxidized by the presence of reactive oxygen species, affecting protein structure and function (7). The enzyme methionine sulfoxide reductase (Msr) can repair oxidized methionine by catalyzing the reduction of methionine sulfoxide residues [Met(O)] back to methionine (Met) in the cytoplasmic methionine pool and in damaged proteins (8). Pathogenic bacteria like *Escherichia coli* (9), *Helicobacter pylori* (10), *Pseudomonas aeruginosa* (11), *Streptococcus pneumoniae* (9, 12), and *Staphylococcus aureus* (13) all contain Msr enzymes that protect against oxidative damage. In *N. gonorrhoeae*, and the closely related bacterial pathogen *Neisseria meningitidis*, the Msr enzyme is a three domain protein named MsrA/B, which contains a thioredoxin-like disulfide reductase, a methionine sulfoxide reductase A and a sulfoxide reductase B (14, 15). MsrA/B of *N. gonorrhoeae* was initially named PilB and was thought to be a pili regulatory protein involved in bacterial adherence to host cells (16). However, later studies disproved this role and showed that the gonococcal MsrA/B is involved in protection from oxidative stress (17), and is upregulated by H_2O_2 (18) and Ecf/sigma E (19). Structural and catalytic studies of MsrA/B revealed its roles in binding and reducing both Met-R(O) and Met-S(O) epimers in *N. gonorrhoeae* (14, 20) and *N. meningitidis* (15, 21–28). The majority of Msr proteins are found in the cytoplasm, where they are collocated with a thioredoxin/thioredoxin reductase system that is involved in regenerating oxidized Msr back to its active form (29). However, based on cell fractionation experiments the gonococcal MsrA/B was proposed to be present in two forms, a truncated inactive cytoplasmic form and a full-length active form located in the outer membrane (17), and presumed to be facing the periplasm (20, 24).

In this study, we examine the conservation and localization of MsrA/B in *N. gonorrhoeae* and *N. meningitidis*. We also investigate the level, type, and functional activity of antibodies raised to the recombinant gonococcal MsrA/B antigen in order to assess its potential as a gonococcal vaccine candidate.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

N. gonorrhoeae 1291 (30), 20 clinical isolates from mucosal and disseminated gonococcal infections (31) and *N. meningitidis* MC58¢3 (32) strains were grown on GC agar (Oxoid) with 1% IsoVitaleX (Becton Dickinson) or Brain Heart Infusion (BHI, Oxoid) 1% agar with 10% Levinthal's Base medium at 37°C with 5% CO_2, respectively, with either kanamycin (kan) (100 μg/ml) or tetracycline (5 μg/ml) as required.

Sequence Bioinformatics Analysis

Distribution of MsrA/B in gonococcal genomes, available at GenBank and at the Meningitis Research Foundation (MRF) Meningococcus Genome Library (PubMLST) database, was investigated using BLAST search with MsrA/B from *N. gonorrhoeae* 1291 (GenBank Accession: protein-EEH61172.1; nucleotide-DS999919.1, Locus tag NGAG_00088).

Generation of Mutant Strains

The 1,569 bp *msr* gene from *N. gonorrhoeae* 1291 was amplified with primers 1291pilBFor and 1291pilBRev (see **Table S1** for primers) containing the *Neisseria* uptake sequence 5′-GCCGTCTGAA-3′ and the resulting PCR product was cloned into pGem ®-T Easy (Promega) to generate pGemT*msr*. The Mutation Generation System™ (MGS) kit (Thermo Fisher) was used according to the manufacturer's instructions to insert a transposon containing a *kanR3* gene into pGemT*msr*. The location and orientation of the *kanR3* in *msr* was determined by sequencing. The *msr::kan* construct was linearized and transformed into *N. gonorrhoeae* 1291 and *N. meningitidis* MC58¢3 by homologous recombination to generate 1291*msr::kan* and MC58¢3*msr::kan* mutant strains. To generate complemented strains, the intact *msr* gene was introduced into either the 1291 *msr::kan* mutant using the complementation plasmid pCTS32 (33), or the MC58¢3 *msr::kan* mutant using pComPind (34).

MsrA/B Protein Expression

The *msr* gene was amplified from *N. gonorrhoeae* 1291 using primers msrexp_NdeIF and msrexp_XhoIR (**Table S1**) and the resulting PCR product was cloned into pET15b to obtain a Msr expression construct pET15bmsr. The construct pET15bmsr was transformed into *E. coli* BL21 Star (DE3)pLysS host strain (Novagen) and MsrA/B was overexpressed and purified. Briefly, expression was induced by 0.1 mM IPTG at an optical density at 600 nm (OD_{600}) of 0.4 for 24 h at room temperature. Cell cultures were harvested and the cell pellet was re-suspended in buffer A. Cells were lysed by sonication, centrifuged, and the supernatant was applied to a column packed with TALON™ metal affinity resins (Clontech Laboratories, Inc.). Unbound proteins were

washed away with 20 column volumes of buffer A, followed by 10 column volumes of buffer A with 20 mM imidazole. The MsrA/B protein was eluted in fractions of 1 ml of 200 mM imidazole. Fractions were collected and analyzed by 4–12% NuPAGE® Novex Bis-Tris Gels (Invitrogen) stained with Coomassie Blue, and by Western blot of anti-His polyclonal antisera.

MsrA/B Mouse Antisera Production (anti-MsrA/B)

Groups of 10 female BALB/c mice (6 weeks old) were immunized subcutaneously with 5 μg of recombinant MsrA/B with either Alhydrogel® (aluminum hydroxide, InvivoGen) or Freund's (FCA/FIA, Sigma-Aldrich) adjuvant on days 0, 21, and 28. Terminal bleeds were collected on day 42. For Freund's adjuvant, Freund's complete adjuvant (FCA) was used on day 0 and Freund's incomplete adjuvant (FIA) was used in the boosts of day 21 and 28. Pre-bleed of each mouse was collected 4 days before immunization. This study was carried out in accordance with the recommendations of the Australian Code for the Care and Use of Animals for Scientific Purposes, the Griffith University Animal Ethics Committee (AEC). The protocol was approved by the Griffith University AEC.

Cell Surface Trypsin Digestion

Overnight culture of 1291 and MC58¢3 were inoculated into appropriate media at an OD_{600} of 0.05. After 2 h growth at 37°C, cells were harvested, washed once, and resuspended in PBS to an OD_{600} of 2. Cell suspension (200 μl) were treated with trypsin (trypsin gold, Promega) for 60 min at 37°C. Cell suspensions at time 0 and at 60 min were taken in triplicate for the determination of colony forming units (CFUs)/ml to confirm cell viability, and were analyzed by Western blot analysis with anti-MsrA/B. Control antibodies used were to surface exposed PorA (NIBSC-UK-EN63QFG) and cytoplasmic GNA2091 (35, 36).

ELISA

For whole cell ELISA, bacteria were grown on BHI or GC plates for 16 h. Cells were harvested and resuspended in PBS at an OD_{600} of 0.2. Microtiter plate wells were filled with 50 μl of the bacterial suspension and dried at room temperature overnight in the laminar flow cabinet. After the bacteria in the dried wells were heat-killed for 1 h in 56°C. For recombinant protein ELISA, wells of plates were coated with 100 ng of purified recombinant MsrA/B protein in 100 μl of coating buffer (0.5 M carbonate/bicarbonate buffer, pH 9.6) for 1 h at room temperature. All ELISAs were performed with mouse pre-immune or MsrA/B immunized sera, and secondary antibody as specified in the results [polyclonal anti-mouse Ig HRP (Dako) or IgG1, IgG2a, IgG2b, IgG3, or IgM HRP (Thermofisher Scientific)]. The substrate TMB (3,3′, 5,5;-tetramethylbenzidine) solution (Thermofisher Scientific) was used as per manufacture's instruction. Equal amount of 1 N hydrochloric acid was added to stop the reaction. Absorbance was read in a TECAN Model Infinite 200 Pro plate reader at 450 nm.

Serum Bactericidal Assay

N. gonorrhoeae 1291 (∼1 × 10^3 CFU) was incubated in serial dilutions of heat-inactivated (56°C, 60 min) anti-MsrA/B sera or pre-immune sera for 15 min at 37°C, after which normal human serum [pre-absorbed with *N. gonorrhoeae* as described previously (37)] was added to a final concentration of 10% (v/v) as a source of complement. The suspension was then incubated at 37°C, 5% CO_2 for 30 min and bacterial CFU determined by plating out serial dilutions. The bactericidal titer is the reciprocal of the lowest antibody dilution, which induced more than 50% killing after 30 min. Statistical significance was calculated using one-way analysis of variance (ANOVA) and Student's *t*-test.

Whole blood from healthy volunteers was collected by venipuncture. For serum, blood was collected in Vacuette Z serum separator tubes (Greiner Bio-One), allowed to clot for 15 min at room temperature then centrifuged for 10 min at 2,000 × *g*. This study was carried out in accordance with the recommendations of the National Statement on Ethical Conduct in Human Research, the Griffith University Human Research Ethics Committee, with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Griffith University Human Research Ethics Committee.

Opsonophagocytic Killing Assay

Polymorphonuclear leukocytes (PMNs) were isolated from donor blood [collected in K3 EDTA tubes (Greiner Bio-One)] using Polymorphprep™ (Axis-Shield) as per manufacturer's instructions, and were resuspended in assay buffer (RPMI (Gibco) supplemented with 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 0.5% (v/v) human serum albumin). *N. gonorrhoeae* 1291 (∼1 × 10^3 CFU) was incubated in serial-dilutions of heat-inactivated anti-MsrA/B sera or pre-immune mouse sera for 15 min at 37°C. PMNs (∼1 × 10^5 cells) and a complement source (10% v/v normal human serum pre-absorbed with *N. gonorrhoeae*) were then added, and incubated at 37°C for 90 min. Gonococcal survival was determined after plating of serial dilutions on GC agar, and survival calculated as a percentage relative to no-antibody control. The opsonophagocytic titer is the reciprocal of the lowest antibody dilution, which induced more than 50% killing after 90 min. Statistical significance was calculated using one-way analysis of variance (ANOVA) and Student's *t*-test.

Surface Plasmon Resonance (SPR)

SPR assays were performed using a Biacore T200 for affinity analysis and a Pall Pioneer FE for competition assays. Affinity assays were performed as previously described (38). Briefly, MsrA/B was immobilized onto flow cell 2 of a Series S CM5 sensor chip using amine coupling kit (GE Life Sciences) at a flow rate of 5 μL/min for 10 min. Flow cell 1 was used as the reference cell and immobilized with ethanolamine only. Met(O) was run at a final concentration range of 0.16–100 nM using single cycle kinetics. Data was analyzed using the Biacore T200 evaluation software package. For competition analysis, MsrA/B was immobilized onto flow cell 1 of a COOH5 Biosensor chip and flow cell 2 the blank immobilized surface using amine coupling

EDC-NHS reactions. Briefly, EDC-NHS mix was flowed at 10 μL/min for 10 min across flow cell 1 and 2. MsrA/B was then flowed across flow cell 1 at 5 μL/min for 20 min in sodium acetate pH 4.5 at a concentration of 25 μg/mL. Ethanolamine was then flowed at 10 μL/min for 10 min to block any remaining active NHS. Competition assays were performed using NextStep injections in the OneStep assay builder. Pre- and post-immune MsrA/B mouse sera were used as the first injection (A), and Met(O) as the second injection (B), with PBS used as a negative control. The competition injection was run for 60 s with the A starting at a 1:100 dilution of serum at time zero and reducing across the injection time, with the B component increasing across the injection reaching 10 μM at 60 s. Binding of Met(O) to MsrA/B was compared with and without serum, and with pre- and post-immune serum. Data was collected using the Pioneer Software package and analyzed using Qdat analysis software. The percentage blocking was calculated based on the relative RMax of the Met(O) injection with and without serum, and the serum with and without Met(O).

RESULTS

MsrA/B Is Highly Conserved in *N. gonorrhoeae* Strains

To investigate the distribution and conservation of MsrA/B in *N. gonorrhoeae* strains, a BLAST search was performed with MsrA/B from *N. gonorrhoeae* 1291 against available genomes. Analysis of *N. gonorrhoeae* genome strains in GenBank revealed that MsrA/B is highly conserved, being present in 100% of 468 strains, with 99–100% amino acid identity over the length of the 522 amino acid protein. An expanded search in the PubMLST database indicated that 86% of gonococcal strains (3,754/4,358) have an annotated *msrAB* gene [NEIS0020 (pilB/msrAB)]. This is likely an underestimate of the presence of *msrAB,* due to the presence of duplicate and incompletely annotated genomes. There are 35 unique gonococcal MsrA/B sequences in the PubMLST database, with 97.5–100% identity between them (**Figure S1**). There are four main variants that are present in 98% of strains, represented by strains PID322 (54% of strains), WHO_K (20%), 1291 (19%), and MS-11 (5%). The *N. gonorrhoeae* 1291 MsrA/B sequence is 98% identical to MsrA/B of *N. meningitidis* MC58 (**Figure S1**).

MsrA/B Is a Surface Protein of the Pathogenic *Neisseria*

N. gonorrhoeae MsrA/B was proposed to be an outer membrane protein based on cell fractionation experiments (17), however the orientation of MsrA/B in the outer membrane was not determined. Our topology prediction analysis using TMHMM (39) indicates that MsrA/B does not have any transmembrane domains and that the whole protein is located outside of the membrane (**Figure S2**). To directly investigate if MsrA/B is surface exposed, whole cell ELISAs of *N. gonorrhoeae* 1291 and *N. meningitidis* MC58¢3 wild-type and *msr::kan* mutant strains were performed with mouse antisera raised against recombinant MsrA/B. Whole cell ELISA indicates that anti-MsrA/B bound to the wild-type 1291 and MC58¢3 intact cells (titer of 256,000 and 512,000, respectively), but binding was significantly reduced to the mutant strains (titer of 8,000 and 1,000, respectively; **Figure 1A**). In addition, MsrA/B was completely susceptible to digestion when intact bacterial cells

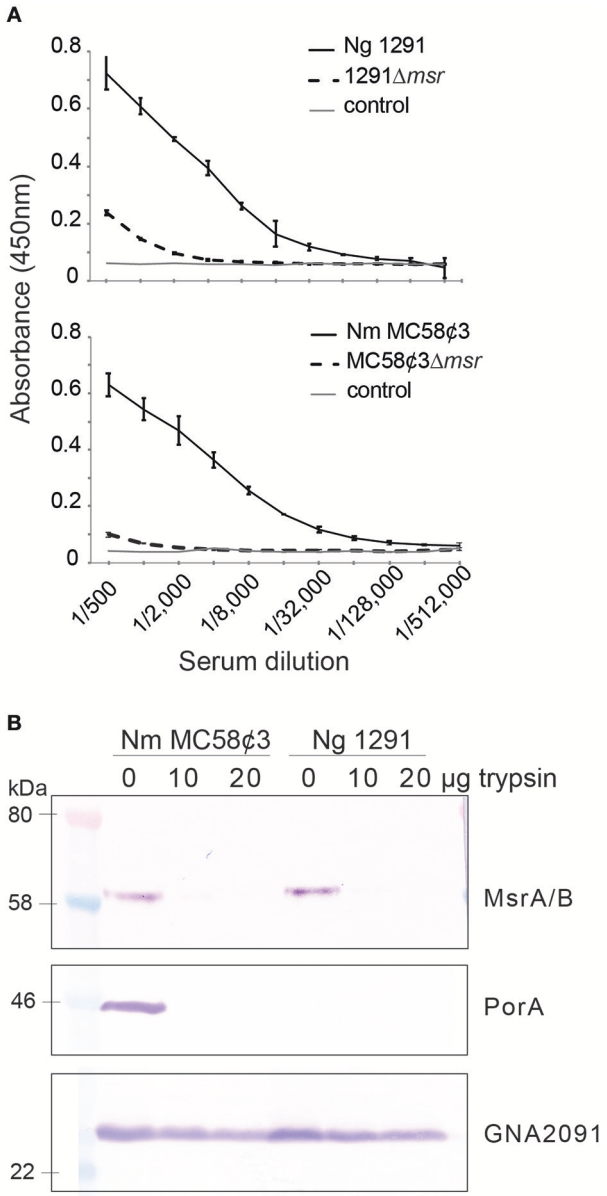

FIGURE 1 | Surface localization of MsrA/B. **(A)** Whole cell ELISA of the wild type (WT) and *msr::kan* mutant (Δ*msr*) strains of *N. gonorrhoeae* 1291 and *N. meningitidis* MC58¢3, with anti-MsrA/B antibodies. The negative control containing secondary antibody only (control), is also shown. The graph shows the average absorbance at 450 nm from three independent replicates, ± one standard deviation. **(B)** Western blot analysis of trypsin treated (20 μg, 10 μg) and untreated (0 μg) whole cell *N. gonorrhoeae* 1291 and *N. meningitidis* MC58¢3, probed with antibodies to MsrA/B, the meningococcal surface protein PorA, and the intracellular protein GNA2091. No significant differences were seen in CFUs/ml at t0 vs. 60 min from samples taken at time 0 and 60 min (two-tailed unpaired Student's *t*-test $p > 1.5$), indicating that no cell lysis occurred during the assay (see **Figure S3**).

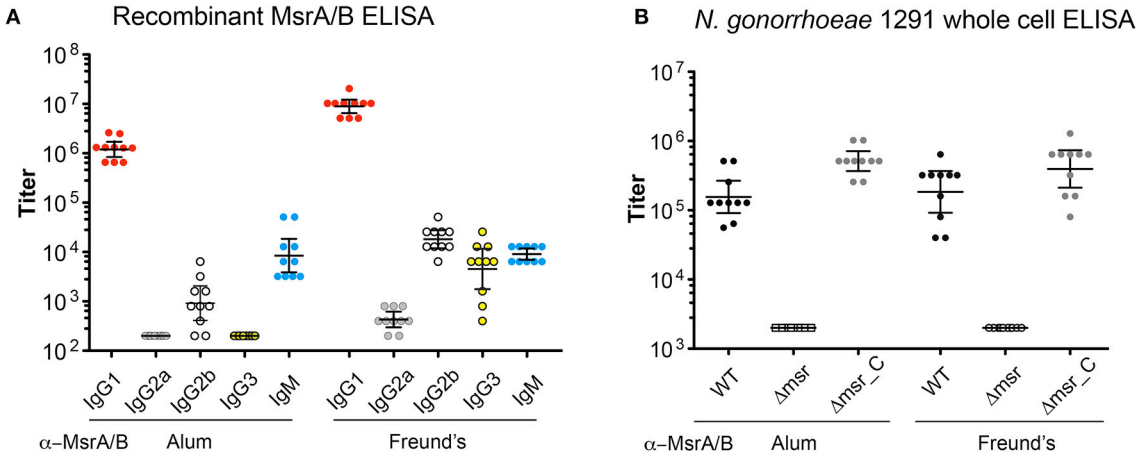

FIGURE 2 | Immunogenicity of MsrA/B. The titers of the post-immune sera from each mouse immunized with either MsrA/B- Alum or MsrA/B-Freund's were determined by ELISA against **(A)** purified recombinant MsrA/B for IgG1, G2a, G2b, G3, IgM, or **(B)** whole cell *N. gonorrhoeae* 1291 wild type (WT), *msr::kan* mutant (Δmsr), and complemented (Δmsr_C) strains for IgG. The titer for each of 10 mice are shown with circles, and the geometric mean titer (GMT) and 95% Confidence interval are indicated bars. The titers of pre-immune sera against whole cell *N. gonorrhoeae* 1291 strains were ≤200.

were treated with 10 or 20 μg of trypsin for 60 min, similar to the meningococcal surface protein PorA (**Figure 1B**, **Figure S3**). The intracellular protein GNA2091 was not affected by trypsin treatment. This ELISA and Western data confirm that MsrA/B is on the surface of both *N. gonorrhoeae* and *N. meningitidis*. Trypsin treatment did not affect cell viability, as there was no significant difference in CFU counts between pre- and post-trypsin treatment (**Figure S3**).

Immunogenicity of Recombinant Gonococcal MsrA/B

To investigate the immunogenicity of MsrA/B, 10 mice were immunized with recombinant MsrA/B with either aluminum hydroxide (MsrA/B-Alum) or Freund's adjuvant (MsrA/B-Freund's). The sera were assessed by ELISA and Western blot. ELISA results with recombinant MsrA/B indicate a dominant IgG1 response in mice immunized with MsrA/B and either adjuvant, with a geometric mean titer (GMT) of 1,222,945 for MsrA/B-Alum and 8,914,438 for MsrA/B-Freund's (**Figure 2A**). Higher titers of IgG2a, IgG2b, and IgG3 were detected in mice immunized MsrA/B-Freund's compared to MsrA/B-Alum, while IgM titers were similar for both adjuvants (**Figure 2A**, **Tables S2**, **S3**).

Whole cell ELISA of the *N. gonorrhoeae* 1291 wild-type, 1291*msr::kan* mutant, and complemented strains indicated that the MsrA/B antisera from each mouse was able to recognize the native MsrA/B protein on the bacterial surface (**Figure 2B**, **Tables S2**, **S3**). There was a similar response against the wild-type from mice immunized with either adjuvant [GMT of 155,496 for MsrA/B-Alum, 183,792 for MsrA/B-Freund's ($p = 0.52$)] and a significantly reduced response to the *msr::kan* mutant strain (GMT of 2,000 for both adjuvant, $p < 0.001$ vs. wild-type). Analysis of MsrA/B-antisera by Western blotting against whole cell lysates of *N. gonorrhoeae* wild-type and the *msr::kan* mutant confirmed that MsrA/B antisera specifically recognize

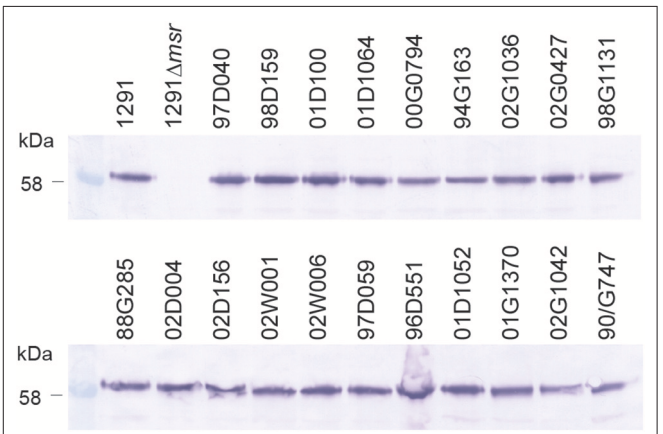

FIGURE 3 | Expression of MsrA/B in a panel of gonococcal strains. Western blot analysis of MsrA/B expression in a panel of *N. gonorrhoeae* strains, including the 1291 wild type and *msr::kan* mutant (1291Δ*msr*), and 20 clinical isolates from mucosal and disseminated gonococcal infections.

MsrA/B. There was no reactivity against MsrA/B in pre-immune sera, while an antibody response was generated by all mice that specifically recognizes MsrA/B in the wild-type strain (**Figure S4**). This ELISA and Western data confirm that MsrA/B is immunogenic and that anti-MsrA/B antisera can specifically recognize MsrA/B on the surface of *N. gonorrhoeae*. The expression of MsrA/B and the cross-reactivity of the MsrA/B antisera was confirmed by Western blot analysis of 20 clinical isolates from mucosal and disseminated gonococcal infections (**Figure 3**, **Figure S5**).

MsrA/B Antisera Has Bactericidal and Opsonophagocytic Activity

Mouse MsrA/B-Alum and MsrA/B-Freund's antisera were investigated for its ability to elicit serum bactericidal activity

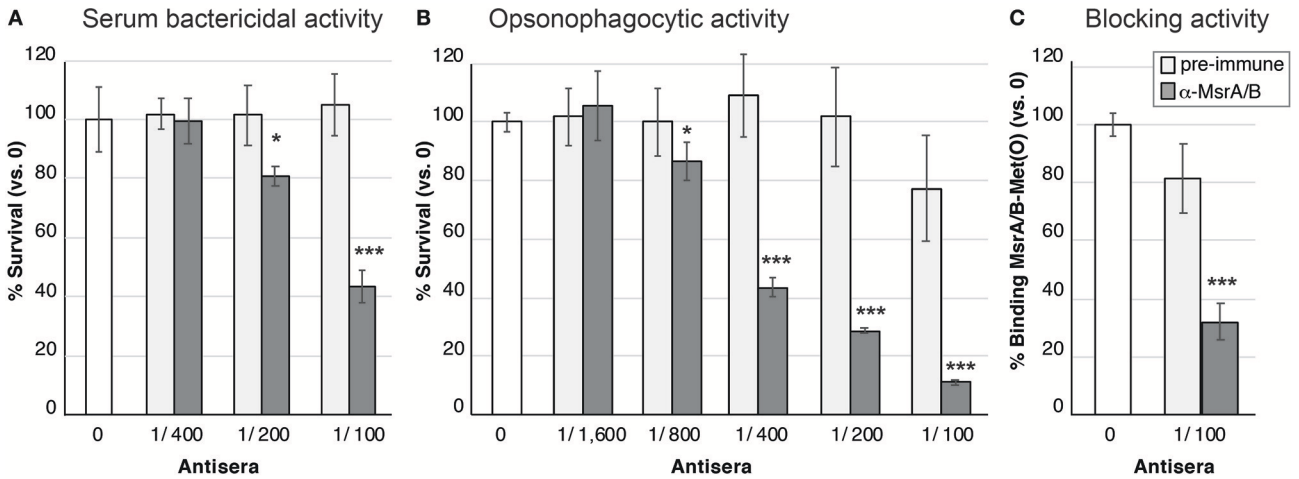

FIGURE 4 | Functional activity of MsrA/B antisera against *N. gonorrhoeae*. **(A)** Serum bactericidal activity. The survival of the *N. gonorrhoeae* in the presence of two-fold dilutions of heat inactivated pre-immune (light gray) or α-MsrA/B (dark gray) sera, plus 10% normal human serum as a complement source is shown. **(B)** Opsonophagocytic activity. The survival of the *N. gonorrhoeae* in the presence of two-fold dilutions of heat inactivated pre-immune (light gray) or α-MsrA/B (dark gray) sera, plus primary human PMNs and 10% normal human serum as a complement source is shown. For **(A,B)**, data represent the mean survival (±1 standard deviation) for triplicate samples, as a percentage of bacteria in the absence of antibody (the no antibody control (white) set at 100%, represents 2.0×10^3 CFU for SBA and 3.5×10^3 CFU for OPA). **(C)** Blocking of MsrA/B binding to Met(O). Surface plasmon resonance (SPR) of MsrA/B interaction with Met(O) was performed in the presence of pre-immune (light gray) or α-MsrA/B (dark gray) sera. Data represents the mean MsrA/B-Met(O) binding (±1 standard deviation) for triplicate samples, as a percentage of MsrA/B-Met(O) binding in the absence of antibody (the no antibody control (white) set at 100%, represents a K_D of 15.4±3.7 nM). For **(A–C)**, statistically significant differences relative to the no serum controls, using a two-tailed Student's *t*-test are indicated: $^*P < 0.05$; $^{***}P \leq 0.001$.

(SBA) and opsonophagocytic (OPA) killing of *N. gonorrhoeae*. Incubation of *N. gonorrhoeae* with serial dilutions of pooled MsrA/B antisera and human serum as the complement source, indicated that MsrA/B-Freund's antisera mediated dose-dependent killing, with an SBA titer of 100 (**Figure 4A**). SBA analysis of MsrA/B-Freund's sera from the 10 individual mice showed dose-dependent killing for 9/10 mice, and a ≥two-fold increase in SBA titer from pre- to post-immune sera for 8/10 mice (**Tables S2**, **S3**). Minimal killing was seen for the MsrA/B-Alum serum at the dilutions tested (titer < 50; **Table S2**).

Incubation of *N. gonorrhoeae* with pooled MsrA/B-Freund's antisera, human PMNs, and human serum as a complement source, revealed dose-dependent opsonophagocytic killing, with a titer of 400 (**Figure 4B**). Analysis of MsrA/B-Freund's serum from the 10 individual mice showed dose-dependent killing, and a ≥two-fold increase in OPA titer from pre- to post-immune sera for 9/10 mice (**Tables S2**, **S3**). The MsrA/B-Alum antisera did not mediate any opsonophagocytic killing (**Tables S2**, **S3**).

MsrA/B Antisera Is Able to Block MsrA/B Binding to Met(O)

To investigate whether MsrA/B antisera was able to block the functional role of MsrA/B, SPR analysis of MsrA/B binding to methionine sulfoxide [Met(O)] was performed in the absence of serum, and in the presence of pre-immune and MsrA/B-Freund's antisera. Recombinant MsrA/B was immobilized on the SPR sensor chip and free Met(O) was flowed over the immobilized protein. MsrA/B bound to Met(O) with a high affinity, with a K_D (equilibrium dissociation constant) of 15.4 ± 3.7 nM (**Figure S6**). A competition assay with pooled MsrA/B-Freund's antisera reduced MsrA/B-Met(O) binding from 100 to 32% (**Figure 4C**; $p \leq 0.002$ vs. no serum or pre-immune sera), while pooled pre-immune sera did not significantly reduce MsrA/B-Met(O) interactions (81 ± 12% binding, $p = 0.05$). Screening of the individual sera showed that 9/10 mice sera significantly blocked MsrA/B-Met(O) binding, compared to no serum and pre-immune serum controls ($p < 0.05$, **Table S2**), with serum from one mouse blocking >99% of MsrA/B binding to Met(O).

DISCUSSION

Antimicrobial resistant *N. gonorrhoeae* is currently an urgent threat to public health worldwide and development of a vaccine is the optimal long-term solution to control gonorrhea [reviewed in Edwards et al. (3)]. Targeting bacterial factors important for survival or virulence is a potential strategy for vaccine development, as a protective immune response against *N. gonorrhoeae* may encompass both functional blocking of human-specific disease processes as well as conventional immune killing processes. The gonococcal methionine sulfoxide reductase MsrA/B plays an important role in protecting *N. gonorrhoeae* from oxidative damage (17), by catalyzing the reduction of methionine sulfoxide residues Met(O) back to methionine (Met) (14, 20). Therefore, we investigated its potential as a gonococcal vaccine antigen. Our results showed that MsrA/B is highly conserved and surface exposed in *N. gonorrhoeae*. Furthermore, we demonstrated that antisera raised to recombinant MsrA/B

mediates bactericidal and opsonophagocytic killing of *N. gonorrhoeae* and is able to inhibit binding of MsrA/B to its substrate, methionine sulfoxide [Met(O)].

The majority of bacterial methionine sulfoxide reductase systems consist of separate cytoplasmic MsrA and MsrB proteins, which are specific for the Met-S(O) and Met-R(O) epimers, respectively. During the catalytic process, firstly a sulfenic intermediate is produced with concurrent release of the repaired Met and, secondly, a recycling step occurs where oxidized MsrA and/or MsrB are reduced to their active form via a thioredoxin/thioredoxin reductase system (29). However, in *N. gonorrhoeae* the MsrA, MsrB and thioredoxin enzymatic functions are present in a single protein, MsrA/B, that is located in the outer membrane (17). MsrA/B was presumed to be facing the periplasmic space, however, we have determined that the gonococcal and meningococcal MsrA/B protein is on the surface. To our knowledge, this is the first time that a Msr has been reported to be surface exposed in a Gram-negative bacterium. In other examples of Gram-negative methionine sulfoxide reductases, the enzyme exists in the cytoplasm and utilizes the cytoplasmic thiol pool regenerated by thioredoxin reductase. It is not clear how MsrA/B of *N. gonorrhoeae* exchanges thiols to maintain activity when it is on the outside of the bacterial cell. A similar problem exists in understanding electron transport with respect to the outer membrane located nitrate reductase AniA, which is active but has no obvious electron donor (40). More work is required to understand how these enzymes function in the context of their localization.

MsrA/B is highly conserved in *N. gonorrhoeae*, with >97% amino acid identity in all strains investigated. Overall, only two sites had common variations: Thr31 substitution to Ala31 in ∼75% of isolates, and Lys520 substitution to Glu520 in ∼25% of isolates. Residue 31 is in the predicted signal peptide of MsrA/B, and all other variant amino acid residues are not located in any known catalytic domains identified in *N. meningitidis* MsrA/B (25, 26, 41). According to Skaar et al. (17), a truncated version of Msr was observed in the cytoplasm and this might due to the miss-cleavage of the signal peptidase. However, we did not see any evidence of a truncated version of MsrA/B in our analysis and believe that the majority of protein is surface exposed. This is not the first example of a gonococcal protein that was found to be surface exposed, despite previously being considered to be a periplasmic protein. For example, both AniA (40) and MetQ (38) are surface exposed on *N. gonorrhoeae*. The AniA nitrite reductase is important for the growth and survival of *N. gonorrhoeae* under oxygen-limited conditions (42) and for biofilm formation (43). Antibodies raised to AniA are capable of functional blocking, able to block AniA nitrite reductase activity of *N. gonorrhoeae* (40). Similarly, MetQ, the methionine binding component of an ABC transporter system is involved in gonococcal adherence to cervical epithelial cells, and antibodies raised to MetQ are able to block gonococcal adherence to epithelial cells and mediate bactericidal activity (38).

MsrA/B was immunogenic in mice when presented with either Alum or Freund's as an adjuvant and antibodies were able to recognize MsrA/B on the surface of *N. gonorrhoeae* via whole cell ELISA. MsrA/B immunization with either Alum and Freund's induced a predominantly IgG1 immune response. Antibodies raised to MsrA/B-Freund's, but not MsrA/B-Alum, mediated killing of *N. gonorrhoeae* via both serum bactericidal activity and opsonophagocytic activity. This activity is probably due to the induction of IgG3 as well as IgG2a and IgG2b by the Freund's adjuvanted MsrA/B antigen. This is consistent with previous findings that mouse IgG isotypes vary in their capacity to activate complement; for example, mouse antibodies targeting PorA of *N. meningitidis* have a hierarchy of IgG3>IgG2>IgG1 for both serum bactericidal and opsonophagocytic activity (44). Anti-MsrA/B-Freund's was also able to block the activity of MsrA/B by inhibiting binding to its substrate. In summary, based on the conservation and immunogenicity of MsrA/B, as well as the functional activity of antibodies raised to it, we propose MsrA/B as a vaccine antigen for *N. gonorrhoeae*.

AUTHOR CONTRIBUTIONS

FJ, ES, and CD performed the experiments. FJ, ES, CD, and KS contributed to writing the manuscript. MJ and KS conceived of the study, directed the collection and analysis of experimental data, edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00137/full#supplementary-material

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