AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

RESEARCH ARTICLE

Variable Expression of Opa Proteins by *Neisseria gonorrhoeae* Influences Bacterial Association and Phagocytic Killing by Human Neutrophils

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ABSTRACT *Neisseria gonorrhoeae* infection is characterized by local and abundant recruitment of neutrophils. Despite neutrophils' antimicrobial activities, viable *N. gonorrhoeae* is recovered from infected individuals, leading to the question of how *N. gonorrhoeae* survives neutrophil attack. One feature impacting *N. gonorrhoeae*-neutrophil interactions is the phase-variable opacity-associated (Opa) proteins. Most Opa proteins engage human carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) to facilitate bacterial binding and invasion. Neutrophils express two transmembrane CEACAMs, CEACAM1 and the granulocyte-specific CEACAM3. While *N. gonorrhoeae* isolated from infected individuals is frequently Opa$^+$, expression of OpaD from strain FA1090, which interacts with CEACAMs 1 and 3, is associated with reduced *N. gonorrhoeae* survival after exposure to human neutrophils. In this study, we hypothesized that the receptor-binding capability of individual Opa proteins impacts bacterial survival in the presence of neutrophils. To test this hypothesis, we introduced *opa* genes that are constitutively expressed into a derivative of strain FA1090 with all 11 *opa* genes deleted. The engineered genes encode Opa proteins that bind CEACAM1 and -3, CEACAM1 but not CEACAM3, or neither CEACAM1 nor -3. *N. gonorrhoeae* expressing CEACAM3-binding Opa proteins survived significantly less well than bacteria expressing other Opa proteins when exposed to primary human neutrophils. The CEACAM3-binding *N. gonorrhoeae* had significantly greater association with and internalization by neutrophils. However, once internalized, bacteria were similarly killed inside neutrophils, regardless of Opa expression. Furthermore, Opa expression did not significantly impact neutrophil granule mobilization. Our findings indicate that the extent to which Opa proteins mediate nonopsonic binding is the predominant determinant of bacterial survival from neutrophils.

IMPORTANCE *Neisseria gonorrhoeae*, the cause of gonorrhea, is an urgent-threat pathogen due to increasing numbers of infections and increased antibiotic resistance. Many surface components of *N. gonorrhoeae* are phase variable, including the Opa protein family of adhesins and invasins. While Opa protein expression is selected for *in vivo*, bacteria expressing some Opa proteins are readily killed by neutrophils, which are recruited to sites of infection. The reason for this discrepancy has remained unresolved. Our work shows that Opa-dependent differences in bacterial survival after exposure to primary human neutrophils correlates with Opa-dependent bacterial binding and phagocytosis. These findings underscore how the ability of *N. gonorrhoeae* to change Opa expression through phase variation contributes to bacterial resistance to neutrophil clearance.

KEYWORDS *Neisseria*, adhesin, binding, invasin, neutrophil, outer membrane protein, phagocytosis, phase variation

Editor Conrad W. Mullineaux, Queen Mary University of London

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The authors declare no conflict of interest.

Received 20 January 2022
Accepted 28 February 2022
Published 28 March 2022

Neisseria gonorrhoeae is the causative agent of the sexually transmitted infection gonorrhea. Gonorrhea is a major public health concern, as there is no protective immunity against future infections, due in part to the variable nature of immunodominant surface antigens (1, 2); for related reasons, there is currently no effective vaccine. Cases of gonorrhea are on the rise, as is resistance to antibiotics, with only ceftriaxone currently recommended for treatment by the U.S. Centers for Disease Control and Prevention (3). These issues emphasize the need to better understand the mechanisms by which *N. gonorrhoeae* infects and successfully colonizes its obligate human host to develop effective new therapeutics and vaccine targets.

N. gonorrhoeae infects mucosal surfaces, including the nasopharynx, rectum, male urethra, and female cervix. Women are more frequently asymptomatic than men and may not seek treatment (4). Despite a robust neutrophil-rich immune response, *N. gonorrhoeae* that is not cleared from infected tissues leads to tissue damage (5). Subsequently, *N. gonorrhoeae* infections that are untreated or escape treatment due to antibiotic resistance can lead to infertility in both sexes and pelvic inflammatory disease and ectopic pregnancy in women.

Neutrophils (the predominant type of polymorphonuclear leukocyte, PMN) are the first line of defense against many invading pathogens, including *N. gonorrhoeae*. Despite the rapid, robust response of neutrophils to many infectious agents, *N. gonorrhoeae* can evade many neutrophil effector functions, including neutrophil extracellular traps, reactive oxygen species (ROS) release, release of antimicrobial peptides and proteases, phagocytosis into degradative compartments, and nutritional immunity (6). Neutrophils recognize *N. gonorrhoeae* through both opsonic and nonopsonic mechanisms, dependent upon the receptor that is interacting with the bacteria (7). In particular, neutrophils phagocytose *N. gonorrhoeae* in a nonopsonic manner using the bacterial surface-expressed Opa proteins (8).

The *opa* family of genes, found in the pathogenic *Neisseria*, encode eight stranded beta barrel proteins that span the outer membrane. Each isolate of *N. gonorrhoeae* carries at least 10 *opa* genes, some of which are duplicates but in distinct chromosomal loci. Each is independently phase variable due to slipped-strand mispairing of a pentameric repeat in the signal sequence-coding portion of the gene. The protein has four extracellular loops, the second and third of which confer the ability of Opa proteins to bind cellular receptors (9). Relative to the rest of the Opa protein, these loops are hypervariable. The hypervariable loops of Opa proteins that bind the same receptors do not always share primary sequence similarity, leading to the assumption that it is the structure and resulting chemical environment of the binding surface, not sequence of the Opa hypervariable loops, that confer receptor binding specificity and selectivity (9). However, these molecular determinants remain to be defined.

While a subset of Opa proteins has been shown to be able to bind to heparan sulfate proteoglycans (HSPGs), the major family of Opa-binding receptors is human carcinoembryonic antigen-related cellular adhesion molecules (CEACAMs). CEACAMs are expressed on multiple cell types relevant to neisserial infection, including neutrophils, endothelial cells, and epithelial cells (10). Human neutrophils express CEACAMs 1, 3, 4, 6, and 8; of these, the neisserial Opa proteins analyzed to date have been shown to interact with CEACAMs 1, 3, and 6 as well as CEACAM5 on epithelial cells (8, 11–13). While CEACAMs 5 and 6 are glycophosphatidylinositol (GPI) anchored, CEACAMs 1 and 3 are transmembrane proteins with cytosolic tails that activate signaling within the cell. CEACAM1 is ubiquitously expressed. Its cytosolic tail contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) that recruits the SHP phosphatases to downregulate cellular activities such as cell proliferation and signaling through the T cell receptor (14, 15). In contrast, CEACAM3, which is restricted to granulocytes, including neutrophils, contains a C-terminal immunoreceptor tyrosine-based activation motif (ITAM). Recruitment of Src family and Syk tyrosine kinases to the CEACAM3 ITAM drives signaling pathways that result in actin-dependent phagocytosis, release of neutrophil granules, and production of reactive oxygen species (ROS) (16, 17). CEACAM3 is thought to

have evolved on primate neutrophils as a way to combat the myriad microorganisms that exploit CEACAM1 as a colonization factor (18).

Opa protein expression is selected for *in vivo*, with specific Opa proteins appearing more often than others in a given strain background. Most *N. gonorrhoeae* bacteria that can be cultured from the secretions of infected individuals express at least one Opa protein (19). In male volunteers inoculated urethrally with predominantly phase-OFF Opa-negative *N. gonorrhoeae*, most bacteria that are recovered from symptomatic individuals express one or more Opa proteins (19). In male individuals urethrally challenged with Opa$^+$ bacteria, Jerse et al. found that over time there was a selection for specific Opa proteins, and, once expressed, that protein stayed expressed from the first positive sample collected until infection was terminated by antibiotic treatment (20). What drives the selection for expression of particular Opa proteins *in vivo* is not yet clear. However, Sintsova et al. found that primary *N. gonorrhoeae* isolates from urethral and cervical infections more frequently expressed Opa proteins that do not have the ability to bind CEACAM3 (21).

Based on this observation and the understanding of the downstream activation of antimicrobial activity upon CEACAM3 binding, we hypothesized that avoidance of CEACAM3 binding confers a survival advantage to *N. gonorrhoeae* when exposed to neutrophils. To this end, we created an isogenic panel of *N. gonorrhoeae* strain FA1090 of different CEACAM-binding profiles, using Opa proteins that are constitutively expressed or are phase varied for expression. These Opa$^+$ bacteria and their Opa-negative counterparts were examined for their survival after infection of primary human neutrophils. Our findings support a model in which the extent of association is the main determinant of *N. gonorrhoeae* resistance to neutrophil killing, where bacteria that do not bind CEACAM3 have a survival advantage.

RESULTS

***N. gonorrhoeae* strains expressing Opa proteins of different receptor-binding profiles differentially activate human neutrophils.** To investigate Opa-dependent interactions of *N. gonorrhoeae* with neutrophils, we established a panel of *N. gonorrhoeae* strains expressing single Opa proteins that are predicted to interact differently with human neutrophils (Fig. 1). Opa proteins were expressed in two genetic backgrounds, each with its own advantages. In one, constitutively expressed, nonvariable (nv) versions of *opa* genes were introduced into a piliated derivative of strain FA1090 in which its 11 *opa* genes were deleted, called Opaless (22). They were introduced into the *opaD* locus and driven by the *opaD* promoter (Opa50$_{nv}$, OpaD$_{nv}$) or into an intergenic site between *aspC* and *lctP* under ectopic control of the *tac-lac* promoter (Opa54$_{nv}$). The other approach used phase-varied ON Opa expressors (OpaA$^+$, OpaF$^+$, and OpaI$^+$) in the piliated FA1090 Δ*opaBEGK* background, in which the four phenotypically translucent *opa* genes of this strain were deleted. In this background, Opa expression can be followed by colony morphology, where each of the remaining Opa proteins confers a particular colony opacity phenotype, as well as Western blotting using monoclonal antibodies specific to each Opa protein of strain FA1090 (20). We confirmed that each of the used strains grew similarly in media (data not shown), as previously reported by our lab (22).

For this study, we investigated three categories of Opa proteins: those interacting with CEACAM1 and CEACAM3, those interacting with CEACAM1 but not CEACAM3, and those that do not interact with either CEACAM1 or -3. Additionally, we assessed the ability of these Opa proteins to interact with the neutrophil-expressed CEACAM6 and epithelium-restricted CEACAM5, the two other CEACAMs that Opa proteins are reported to bind. Receptor binding was determined by the ability of *N. gonorrhoeae* expressing defined Opa proteins to precipitate the soluble N-terminal domain of each of these CEACAMs (N-CEACAMs), analyzed by imaging flow cytometry (23). Results are reported as both the percentage of Alexa Fluor 488$^+$ (CEACAM-binding) *N. gonorrhoeae* (Fig. 1A) and the mean Alexa Fluor 488 fluorescence intensity of the population (Fig. 1B).

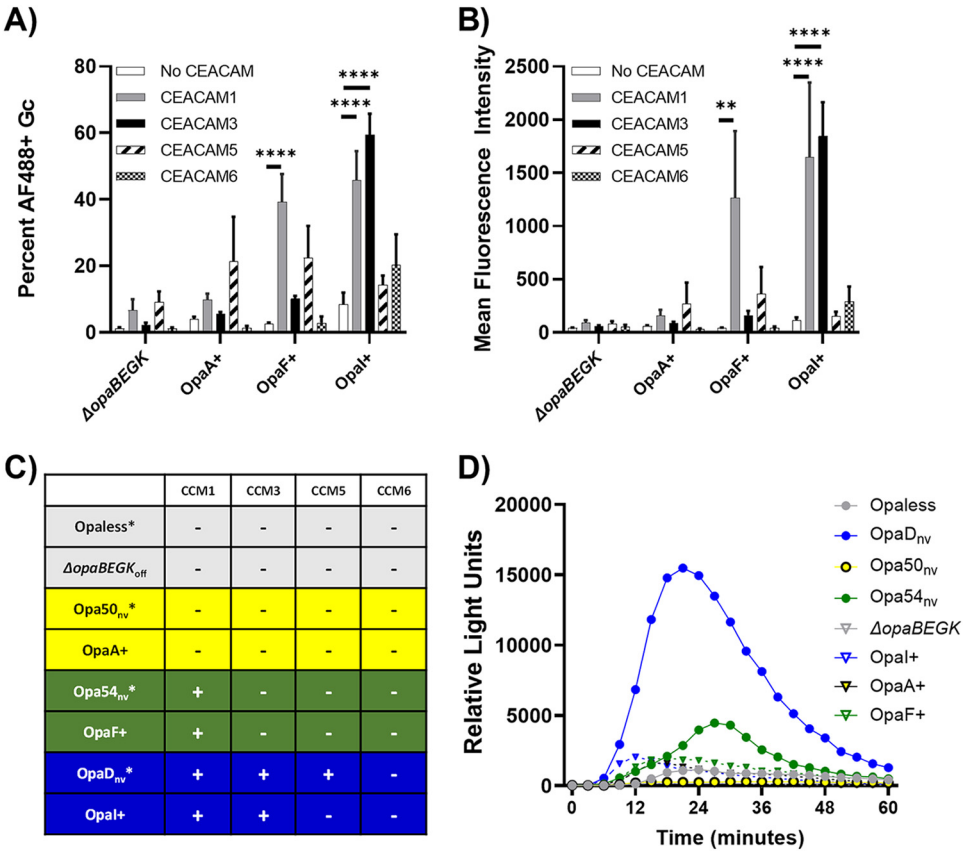

FIG 1 Receptor-binding profile of selected Opa proteins and elicitation of the neutrophil oxidative burst. (A and B) *N. gonorrhoeae* predominantly expressing OpaA, OpaF, or OpaI in the Δ*opaBEGK* background, or the Opa$^-$ control, was incubated with GST-tagged recombinant N-CEACAM1 (gray), N-CEACAM3 (black), N-CEACAM5 (hatched), N-CEACAM6 (checked), or no protein as a control (white). Binding of CEACAM was recognized with an anti-GST antibody followed by Alexa Fluor 488-coupled goat anti-mouse IgG. The capacity of each *N. gonorrhoeae* strain used in this study to bind each CEACAM was determined by imaging flow cytometry. The percentage of the singlet bacterial population in the Alexa Fluor 488$^+$ gate (A) and the mean fluorescence intensity (MFI) of Alexa Fluor 488 (B) were quantified. (C) Data are compiled from panel A and reference 23 (asterisks). Yellow, blue, and green colors are kept consistent throughout this study. Opa$^+$ indicates phase-variable strains, and nv indicates non-phase-variable, locked-ON strains. (D) The indicated strains of *N. gonorrhoeae* at an MOI of 100 were exposed to primary human neutrophils in the presence of luminol. Production of reactive oxygen species was measured as relative light units of luminol-dependent chemiluminescence over 60 min. Circles denote non-phase-variable, locked strains of *N. gonorrhoeae* in Opaless; triangles indicate predominantly phase-ON or -OFF *N. gonorrhoeae* in the Δ*opaBEGK* background (Opa$^-$ bacteria are gray).

The binding capabilities of the nonvariable strains used in this study have been previously reported by our group using the imaging flow cytometry binding assay (23). FA1090 OpaD binds to the N-domains of human CEACAM1, CEACAM3, and CEACAM5 but not CEACAM6, Opa54 of strain MS11 binds to CEACAM1 but not CEACAMs 3, 5, or 6, and Opa50 of strain MS11 does not interact with any CEACAMs (23). These data are consistent with prior reports about Opa54's CEACAM-binding preferences (21, 23) and that Opa50 interacts with heparan sulfate proteoglycans and not CEACAMs (8). Applying the imaging flow cytometry binding assay to the predominantly phase-ON Opa$^+$ *N. gonorrhoeae*, we found that FA1090 OpaI binds to both CEACAMs 1 and 3, OpaF of strain FA1090 interacts with CEACAM1 but not CEACAM3, and OpaA of strain FA1090 does not bind any CEACAMs (Fig. 1A and B). None of the newly tested Opa proteins bound to CEACAM5 or CEACAM6 (Fig. 1A and B). The nucleotide (see Fig. S1A in the supplemental material) and amino acid (Fig. S1B) sequences of each Opa used in this study are reported. The CEACAM binding profiles and genetic background for each strain in this study are presented in Fig. 1B. Opaless and Δ*opaBEGK*$_{off}$ (Δ*opaBEGK*) are the non-Opa-expressing bacteria used throughout this study that did not interact with any CEACAMs (Fig. 1A and B) (23).

To begin to assess how expression of the different Opa proteins affects interaction with primary human neutrophils, we measured neutrophil production of reactive oxygen species (ROS) after exposure to equivalent numbers of CFU of *N. gonorrhoeae*, using luminol-dependent chemiluminescence. ROS production is a consequence of granule trafficking and cytoplasmic signaling events that result in assembly of NADPH oxidase, which produces superoxide and hydrogen peroxide, and myeloperoxidase, which uses hydrogen peroxide to generate hypochlorous acid (24). ROS does not directly contribute to neutrophil antimicrobial activity against *N. gonorrhoeae* (25, 26) but does reflect the activation state of neutrophils in response to infection. CEACAM1 and -3 binding $OpaD_{nv}$ and $OpaI^+$ elicited a rapid (within 15 min) ROS response from neutrophils (Fig. 1D, blue lines). In contrast, the ROS response was slower (peak, ~20 to 30 min) in response to the CEACAM1-only binder $Opa54_{nv}$. $OpaF^+$ *N. gonorrhoeae*, which also binds CEACAM1 and not CEACAM3, elicited a marginal ROS response from neutrophils, with a peak within 15 to 20 min of exposure (green lines with triangles). The non-CEACAM binding strains of *N. gonorrhoeae* elicited minimal release of ROS from neutrophils, similar to the Opaless and Δ*opaBEGK* backgrounds (yellow lines and gray lines). These findings indicate that Opa expression state affects gonococcal activation of neutrophils. In particular, bacteria that bind both CEACAM1 and CEACAM3 tended to stimulate a more rapid and/or potent oxidative response.

CEACAM3 engagement is associated with increased bacterial binding and phagocytosis by neutrophils. We next tested how expression of different Opa proteins affected bacterial association with and phagocytosis by primary human neutrophils. To do so, we applied an imaging flow cytometry assay, which uses a spot count algorithm to quantify the association and phagocytosis of *N. gonorrhoeae* across tens of thousands of neutrophils per condition (27). These experiments used adherent, IL-8-treated primary human neutrophils as a surrogate for neutrophils that have migrated to sites of mucosal *N. gonorrhoeae* infection (28).

At 15 min postinfection at a multiplicity of infection (MOI) of 1, fewer than 20% of neutrophils were associated with the non-CEACAM or CEACAM1 only binding nonvariable *N. gonorrhoeae* (average for Opaless, 4.4%; $Opa50_{nv}$, 7.7%; $Opa54_{nv}$, 12%) (Fig. 2A). In contrast, at the same time point, CEACAM1 and CEACAM3 binding $OpaD_{nv}$ showed significantly more association with neutrophils ($OpaD_{nv}$, 52%) (Fig. 2A). Similarly, at 30 min and 60 min, neutrophils were significantly more associated with $OpaD_{nv}$ *N. gonorrhoeae* than with Opaless, $Opa50_{nv}$, or $Opa54_{nv}$ bacteria (Fig. 2A). At 60 min, significantly more neutrophils were also associated with $Opa50_{nv}$ than Opaless.

The phase-varied-ON strains followed similar patterns (Fig. 2B). At 15 and 30 min, $OpaF^+$ and $OpaI^+$ *N. gonorrhoeae* showed higher association with neutrophils than $OpaA^+$ or Δ*opaBEGK* bacteria, but these differences were not statistically significant (Δ*opaBEGK*, 14%; $OpaA^+$, 7.7%; $OpaF^+$, 31%; $OpaI^+$, 32%). By 60 min, $OpaI^+$ *N. gonorrhoeae* was associated with more neutrophils than the other strains and was significantly increased over Δ*opaBEGK* bacteria (Fig. 2B). A histogram of the number of *N. gonorrhoeae* cells counted per neutrophil in each population is presented in Fig. S2.

The same data set was analyzed for bacterial phagocytosis, with the output being the percentage of neutrophils with intracellular *N. gonorrhoeae* (see Materials and Methods for details). Significantly more neutrophils contained intracellular $OpaD_{nv}$ *N. gonorrhoeae* than the other nonvariable bacteria at all measured time points postinfection (Fig. 2C). There were no significant differences between Opaless and $Opa50_{nv}$ or $Opa54_{nv}$ at any of the time points. Similar to the percent association data for the phase-varied-ON *N. gonorrhoeae*, neutrophils internalized more $OpaI^+$ than $OpaA^+$ or Δ*opaBEGK* strain at 30 and 60 min postinfection, with the difference between $OpaI^+$ and $OpaA^+$ being statistically significant at both time points and the difference between $OpaI^+$ and Δ*opaBEGK* strain being statistically significant at 60 min (Fig. 2D). While there was a trend toward more phagocytosis of $OpaI^+$ than $OpaF^+$ *N. gonorrhoeae* as time progressed, this was not statistically significant.

The results with imaging flow cytometry were extended using an immunofluorescence assay that reports the percentage of cell-associated bacteria that are intracellular (28).

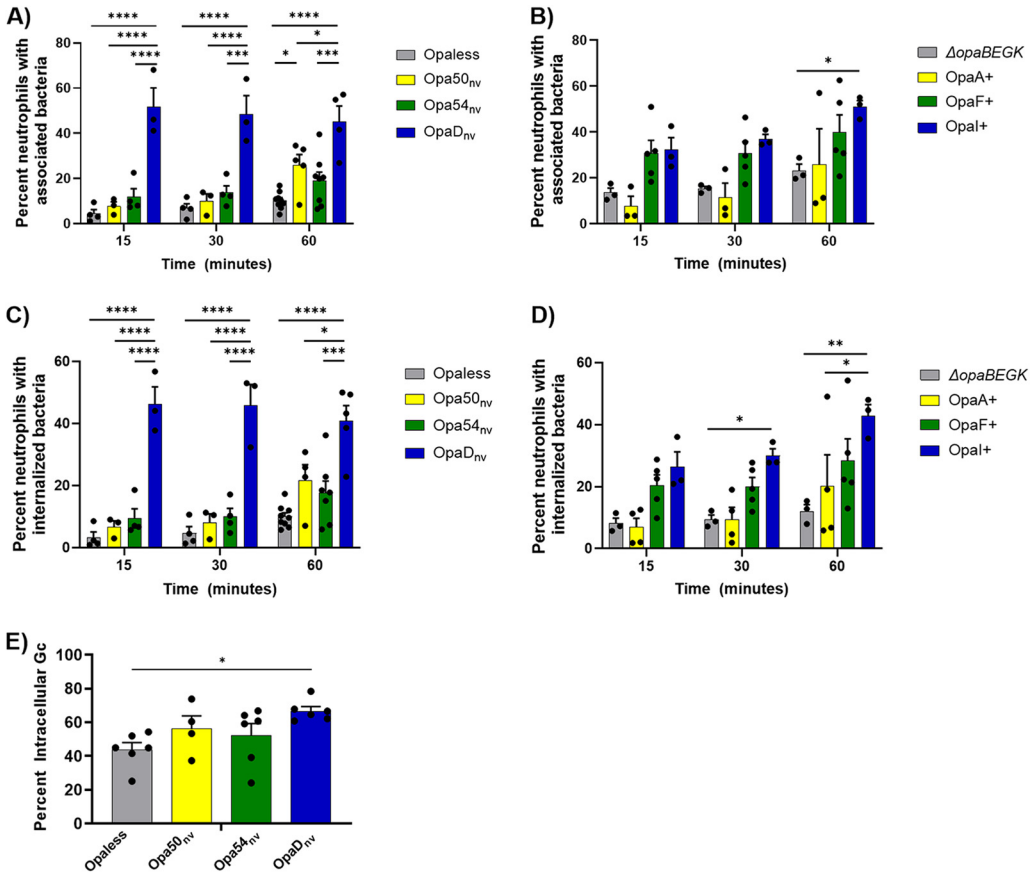

FIG 2 Expression of different Opa proteins differentially affects binding and phagocytosis of *N. gonorrhoeae* by primary human neutrophils. The indicated strains of *N. gonorrhoeae* (A and C, constitutively expressed, nonvariable; B and D, phase variable) were labeled with Tag-IT Violet (TIV) and incubated with adherent, IL-8-treated primary human neutrophils. At the indicated times, cells were fixed and stained with DyLight 650 (DL650)-labeled anti-*N. gonorrhoeae* antibody without permeabilization to recognize extracellular bacteria. Neutrophils were analyzed via imaging flow cytometry. Panels A and B report the percentage of single, intact neutrophils with ≥1 cell-associated bacterium (TIV^+). Panels C and D indicate the percentage of neutrophils with ≥1 phagocytosed bacterium (TIV^+ $DL650^-$). Results are the average of $n \geq 3$ biological replicates. Data were analyzed by two-way ANOVA with Tukey's multiple comparisons, with the following indications of significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; ****, $P < 0.001$. Only statistical comparisons within a time point were made. (E) The indicated strains of *N. gonorrhoeae* were labeled with CFSE and then incubated with adherent, IL-8-treated neutrophils. After 60 min, cells were fixed and stained with AlexaFluor 647 (AF647)-labeled anti-bacteria antibody without permeabilization. Images were captured by fluorescence microscopy. The percentage of intracellular *N. gonorrhoeae* was determined by dividing the number of $CFSE^+$ $AF647^-$ (intracellular) *N. gonorrhoeae* by the number of $CFSE^+$ $AF647^+$ (total) *N. gonorrhoeae*. Statistical comparisons were made for $n \geq 4$ biological replicates using one-way ANOVA with Tukey's multiple comparisons, with $P < 0.05$ (*) considered significant.

Here, we focused on the nonvariable Opa strains so that the potential confounder of phase variation was removed. At 60 min postinfection, there were no significant differences among the Opa-expressing *N. gonorrhoeae* strains in the percentage of neutrophil-associated bacteria that were phagocytosed ($Opa50_{nv}$, 56%; $Opa54_{nv}$, 52%; $OpaD_{nv}$, 67%) (Fig. 2E). However, the percentage of intracellular Opaless bacteria (44%) was significantly lower than that for $OpaD_{nv}$, in keeping with prior reports (Fig. 2E) (29, 30).

Taken together, these results indicate that *N. gonorrhoeae* expressing different Opa proteins differentially interacts with human neutrophils. However, once bound to neutrophils, Opa^+ *N. gonorrhoeae* is readily phagocytosed, regardless of which receptor(s) it engages.

Survival of *N. gonorrhoeae* from primary human neutrophils is modulated by bacterial Opa expression profile. We examined how the association and phagocytosis of different Opa-expressing *N. gonorrhoeae* strains affected bacterial survival from neutrophils by enumerating CFU of bacteria from neutrophil lysates as a function of time (31). First examining the nonvariable strains, similar CFU numbers of Opaless,

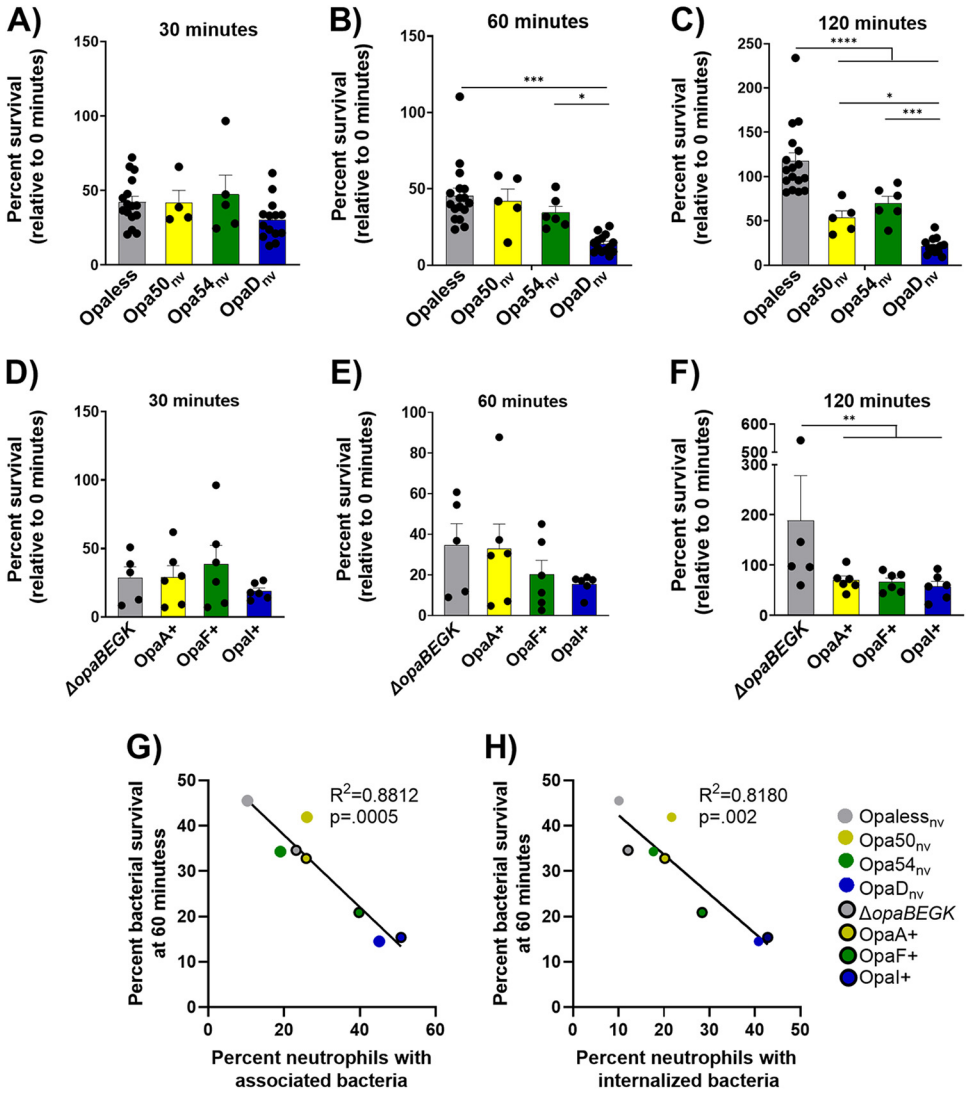

FIG 3 Differential survival of *N. gonorrhoeae* of different Opa expression states after exposure to primary human neutrophils. Adherent, IL-8-treated neutrophils were synchronously exposed to constitutively expressed, nonvariable (A to C), or phase-variable (D to F) *N. gonorrhoeae* of the indicated Opa profile. Colors match the receptor-binding profile of each strain as in Fig. 1B. At 30 (A and D), 60 (B and E), and 120 (C and F) min postinfection, neutrophils were lysed and CFU of *N. gonorrhoeae* were enumerated from the lysates. Results are expressed as the average percentage of CFU at that time point divided by the CFU at the start of the experiment (0 min) ± standard errors for $n \geq 4$ biological replicates. Statistical comparisons were by two-way ANOVA with *post hoc* Tukey multiple-comparison test, with the following pairwise significances: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; ****, $P < 0.0001$. Separate ANOVAs were run to compare the variable strains and the nonvariable strains. Correlations between bacterial survival and association with neutrophils (G) and bacterial survival and phagocytosis by neutrophils (H) were calculated using a linear regression model. The R^2 and P values are reported on the graphs.

$OpaD_{nv}$, $Opa54_{nv}$, and $Opa50_{nv}$ were recovered after 30 min of neutrophil exposure (Fig. 3A). However, after 60 min, fewer CFU of $OpaD_{nv}$ *N. gonorrhoeae* were recovered than with any of the other strains, with the differences from Opaless and $Opa54_{nv}$ *N. gonorrhoeae* being statistically significant (Fig. 3B). By 120 min postinfection, the recovery of $OpaD_{nv}$ *N. gonorrhoeae* was significantly less than that of any of the other comparator strains (Fig. 3C). We also saw outgrowth of Opaless at 120 min postinfection, likely due to extracellular replication of the bacteria; the difference between Opaless and all of the Opa-expressing strains was statistically significant at this time point.

The phase-varied-ON strains of *N. gonorrhoeae* followed the same trends as the Opa-nonvariable strains across all the time points tested, with no differences among

Δ*opaBEGK*, OpaA$^+$, OpaF$^+$, and OpaI$^+$ strains at 30 min (Fig. 3D). While more CFU of Δ*opaBEGK* and OpaA$^+$ *N. gonorrhoeae* strains were recovered at 60 min than with OpaF$^+$ or OpaI$^+$ bacteria, these differences were not statistically significant (Fig. 3E). By 120 min, Δ*opaBEGK* bacteria were recovered in significantly greater numbers than any of the other Opa$^+$ bacteria (Fig. 3F). Interestingly, the CEACAM-binding profile did not directly correlate with bacterial susceptibility to neutrophils: while the CEACAM1 and CEACAM3 binding OpaD$_{nv}$ strain survived significantly less well than any of its direct comparators, the OpaI$^+$ strain, which also binds CEACAM1 and CEACAM3, survived similarly to the other Opa expressors. Overall, we saw an inverse correlation between association with neutrophils and survival of the bacteria as well as internalization into neutrophils and survival of bacteria (Fig. 3G and H).

Opa-receptor interactions do not affect the survival or maturity of phagosomes containing Opa$^\pm$ *N. gonorrhoeae* inside human neutrophils. Having observed a correlation between bacterial association with neutrophils and their resistance to neutrophil-mediated killing, we examined but ruled out other possibilities that could explain the difference in Opa$^+$ *N. gonorrhoeae* survival from neutrophils. First, we found that Opa-expressing *N. gonorrhoeae*, once phagocytosed, exhibited similarly low intracellular survival in neutrophils, as determined based on their permeability to propidium iodide (32). Extracellular *N. gonorrhoeae* exhibited greater viability than intracellular bacteria, as previously reported (30), but there was no measurable difference in viability based on Opa expression in either the intracellular (Fig. 4A) or the extracellular (Fig. 4B) compartment. Next, we evaluated the maturity of the phagosome in which the phagocytosed *N. gonorrhoeae* was found based on acquisition of the primary granule protein neutrophil elastase (30). At 60 min, all Opa$^+$ *N. gonorrhoeae* bacteria, regardless of receptor-binding profile, resided in phagosomes of similar maturity (Fig. 4C), with a trend toward reduced phagosome maturation for Opaless *N. gonorrhoeae*, as previously reported ($P = 0.08$) (30). There was also no significant difference in primary granule exocytosis in response to exposure to the different Opa$^+$ *N. gonorrhoeae* variants, as reported by mean CD63 fluorescence intensity normalized to Opaless using flow cytometry (Fig. 4D).

Taken together, we conclude that avoiding association is the major route by which *N. gonorrhoeae* survives exposure to neutrophils. For a given bacterial strain, the *N. gonorrhoeae* bacteria that are most susceptible to killing by neutrophils express Opa proteins that most strongly increase neutrophil binding and phagocytosis.

Outgrowth of Opa-negative gonococci in a population of Opa-phase-ON bacteria that highly associate with neutrophils. We were surprised to measure an increase in CFU numbers of OpaI$^+$ bacteria recovered from neutrophils at 120 min, relative to 30 min and 60 min (Fig. 3F), given that OpaI binds to CEACAMs 1 and 3 and is readily bound and phagocytosed by neutrophils (Fig. 2C and D). To examine this Opa expressor further, we generated a strain of *N. gonorrhoeae* with a constitutively expressed, nonvariable *opaI* in the OpaD locus (OpaI$_{nv}$) (see Materials and Methods for details). While similar CFU counts of OpaI$_{nv}$ and OpaI$^+$ *N. gonorrhoeae* were enumerated at earlier times of infection, at 120 min significantly fewer OpaI$_{nv}$ were recovered than OpaI$^+$ bacteria (Fig. 5A). We hypothesized that this discrepancy was due to the phase variability of OpaI in this background. To test this, we quantified the opacity-related morphology of the colonies from the OpaI$^+$ population after exposure to neutrophils and in the medium control (RPMI with 10% fetal bovine serum [FBS]). The percentage of Opa$^+$ colonies in the OpaI$^+$ inoculum (zero minutes) with neutrophils was similar to the percentage in the medium control, as expected (Fig. 5B and C). However, in the presence of neutrophils, the percentage of Opa$^+$ colonies in the population significantly decreased over time, such that by 120 min, 72% of the colonies associated with neutrophils were Opa$^-$ (Fig. 5B). In the medium control, 76% of the starting OpaI$^+$ population was Opa$^+$, and this percentage did not significantly change over time (Fig. 5C). Within the OpaI$^+$ population, greater numbers of Opa$^-$ (OpaI phase OFF) than Opa-expressing bacteria were recovered over time from neutrophils. This increase in the Opa$^-$ bacteria over time resulted in an increase in the overall survival of the OpaI$^+$ population at 120 min of neutrophil exposure (Fig. S3A).

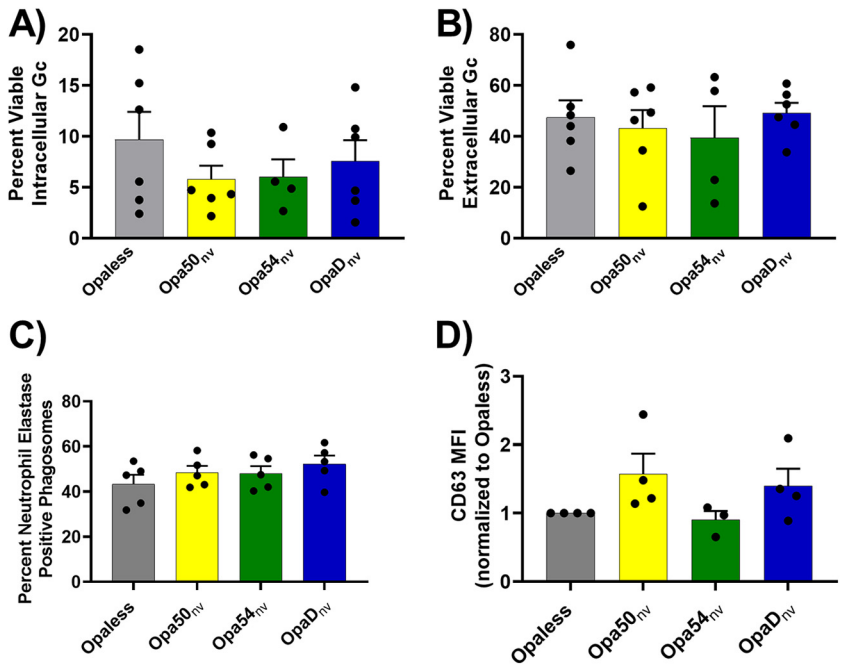

FIG 4 Opa expression state does not affect viability of *N. gonorrhoeae* in the intracellular or extracellular compartments of neutrophils or the release of neutrophil primary granules. (A and B) *N. gonorrhoeae* was incubated with adherent, IL-8-treated primary human neutrophils for 1 h. Infected neutrophils were exposed to AF647-coupled soybean lectin to recognize extracellular *N. gonorrhoeae* and then exposed to BacLight LIVE/DEAD viability dyes in the presence of saponin. The percentage of viable (SYTO9$^+$) *N. gonorrhoeae* in the intracellular (AF647$^-$) (A) and extracellular (AF647$^+$) (B) compartments was quantified for $n \geq 4$ biological replicates. There were no statistical differences among strains in either compartment using one-way ANOVA with Tukey's multiple-comparison test. (C) Adherent, IL-8-treated neutrophils were exposed to CFSE-labeled *N. gonorrhoeae* for 60 min. Cells were fixed and stained without permeabilization with rabbit anti-*N. gonorrhoeae* antibody, followed by AF647-coupled goat anti-rabbit IgG, to label extracellular *N. gonorrhoeae*. Cells were refixed and exposed to mouse antineutrophil elastase IgG followed by Alexa Fluor 555-coupled goat anti-mouse IgG. The percentage of intracellular (CFSE$^+$ AF647$^-$) *N. gonorrhoeae* in neutrophil elastase-positive phagosomes was quantified. Results are from $n \geq 3$ biological replicates. There were no statistically significant differences by one-way ANOVA with Tukey's multiple-comparison test. (D) Adherent, IL-8-treated neutrophils were exposed to the indicated strains of *N. gonorrhoeae* for 60 min. Neutrophils were analyzed for the presence of the primary granule protein CD63 on the cell surface by flow cytometry. Data are presented as the mean fluorescence intensity (MFI) of CD63 and expressed relative to Opaless to account for human subject-intrinsic variability in CD63 expression. Results are from $n \geq 3$ biological replicates. Shapes indicate individual matched data points from each experiment. There were no statistically significant differences by one-way ANOVA with Tukey's multiple-comparison test.

We asked if the same observation would be made for OpaD-expressing bacteria, which also highly associated with neutrophils (Fig. 2A and B) and survived less well from neutrophils than the other Opa-nonvariable bacteria (Fig. 3A to C). To do so, we isolated OpaD phase-ON (OpaD$^+$) bacteria from the Δ*opaBEGK* background and compared their survival to that of OpaD$_{nv}$ after exposure to neutrophils. OpaD$^+$ trended toward surviving better than OpaD$_{nv}$ from neutrophils but was not statistically significantly different (Fig. 5D). Similar to OpaI$^+$, there was a shift in the OpaD$^+$ population after exposure to neutrophils, with significantly greater numbers of Opa phase-OFF bacteria by 120 min (77% Opa$^+$ in the inoculum versus 55% at 120 min) (Fig. 5E). OpaD$^+$ in the medium control maintained its Opa$^+$ status over time (Fig. 5F). As with OpaI$^+$, the Opa$^-$ bacteria outgrew the OpaD$^+$ bacteria, skewing the survival of the OpaD$^+$ population higher than what was seen for OpaD$_{nv}$ (Fig. S3B).

Similar analyses were performed for the parent of the variable strains, Δ*opaBEGK* strain, and the OpaA$^+$ and OpaF$^+$ expressors. After exposure to neutrophils, >95% of Δ*opaBEGK* colonies remained Opa$^-$ throughout the 120-min infection period (Fig. 5G). While there was a slight increase over time in the proportion of Opa$^-$ colonies in the

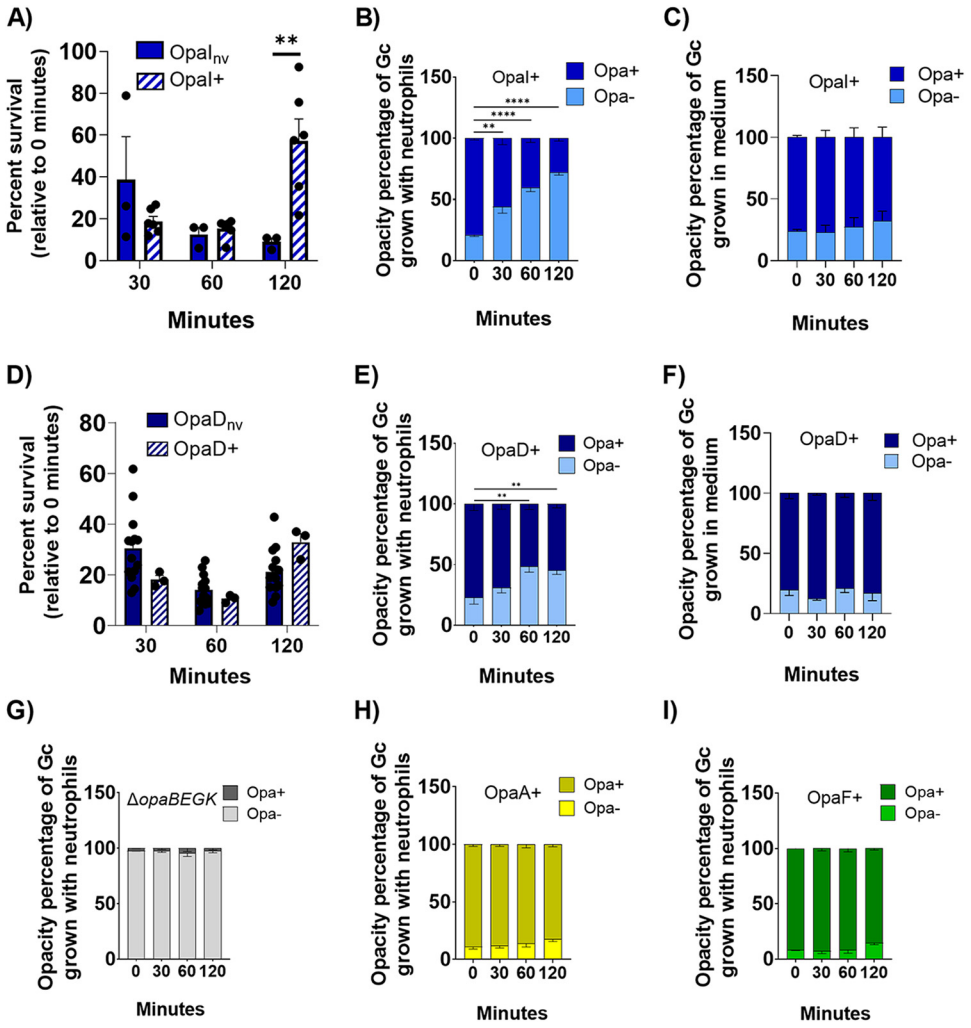

FIG 5 Neutrophil challenge selects for phase-OFF expression of Opa proteins that drive association of *N. gonorrhoeae* with neutrophils, increasing overall survival of the population of *N. gonorrhoeae*. *N. gonorrhoeae* bacteria that were constitutively expressing (nv) or phase-ON ($^+$) for Opal (A) or OpaD (D) were exposed to adherent, IL-8-treated primary human neutrophils, and CFU were enumerated from bacterial lysates over time as for Fig. 3. Bacterial survival is expressed relative to the CFU enumerated at time zero. At each time point from panels A and D, opacity phenotype of the enumerated CFU was visually inspected and recorded. Results are reported as the percentage of colonies at the indicated time point that retained Opa expression (dark bars) or were Opa negative (light bars). No other opacity phenotypes other than the indicated Opal$^+$ (B) and OpaD$^+$ (E) were observed. The same starting cultures of Opal$^+$ (B) and OpaD$^+$ (E) as above were inoculated into media without neutrophils, and CFU of Opa$^-$ and Opa$^+$ phenotypes were enumerated and plotted. No change was seen for with Opal$^+$ (C) or OpaD$^+$ (F). Δ*opaBEGK* (G), OpaA$^+$ (H), and OpaF$^+$ (I) *N. gonorrhoeae* strains were exposed to neutrophils, and the opacity phenotypes of the CFU recovered at each time point were plotted as in panels B and E. Statistical comparisons were by two-way ANOVA with *post hoc* Tukey multiple-comparison test, with the following pairwise significances: *, $P < 0.05$, **, $P < 0.01$, ****, $P < 0.0001$.

OpaA$^+$ (Fig. 5H) and OpaF$^+$ (Fig. 5I) populations after exposure to neutrophils, this change was not statistically significant. Expression of other Opa proteins in the OpaA$^+$ and OpaF$^+$ backgrounds was not noted, as judged by colony opacity phenotype. There was no significant change in Opa expression state for these bacteria in the medium control, with Δ*opaBEGK* strain remaining predominantly Opa$^-$ and OpaA$^+$ and OpaF$^+$ and mostly Opa$^+$ (Fig. S4A, B, and C). While there tended to be more Opa$^-$ than Opa$^+$ colonies in these variable Opa populations 120 min postneutrophil infection, this change did not have a major impact on the overall survival of the Δ*opaBEGK*, OpaA$^+$, and OpaF$^+$ populations (Fig. S3C to E).

Taken together, these data indicate that the ability to phase vary Opa expression is advantageous for *N. gonorrhoeae*. A heterogeneous population allows the bacteria to

avoid phagocytic killing by neutrophils, specifically in cases where the Opa$^+$ bacteria are rapidly and efficiently phagocytosed.

DISCUSSION

Opa proteins are an important family of adhesins and invasins for *N. gonorrhoeae*, and most *N. gonorrhoeae* bacteria isolated from individuals with symptomatic uncomplicated gonorrhea are phenotypically Opa$^+$. However, we and others have found that Opa$^+$ bacteria are more susceptible to killing by neutrophils than those lacking Opa expression. In this work, we investigated how differential interaction with primary human neutrophils affects *N. gonorrhoeae* survival in an Opa-dependent manner. To do so, we used two panels of isogenic *N. gonorrhoeae*, one with or without constitutive expression of a single Opa and the other with single phase-varied-ON Opa$^+$ bacteria and defined the receptor-binding profile of the Opa expressors. We found that Opa expression alone does not dictate the survival of *N. gonorrhoeae* after exposure to adherent, IL-8-treated primary human neutrophils. Instead, survival is impacted by the degree to which Opa expression affects *N. gonorrhoeae* association with and phagocytosis by neutrophils. In particular, bacteria expressing Opa proteins that do not bind to the granulocyte-specific CEACAM3 were more successful at avoiding phagocytosis and killing by neutrophils. While the phase-variable nature of Opa proteins makes them a less than ideal vaccine target, our results suggest therapeutics that promote the phagocytic killing activities of locally recruited neutrophils would be effective at combating *N. gonorrhoeae*, regardless of which Opa protein(s) the bacteria in the population expresses.

For this study, we examined six Opa proteins, two that do not interact with neutrophils via CEACAMs (OpaA of FA1090 and Opa50 of MS11), two that interact via CEACAM1 (OpaF of FA1090 and Opa54 of MS11), and two that interact via both CEACAM1 and CEACAM3 (OpaI and OpaD of FA1090), all in the FA1090 strain background. None of the Opa proteins in this study were found to interact with CEACAM6, and CEACAM5, which is bound by OpaD, is not expressed by neutrophils. The use of both phase-variable and nonvariable strains conferred advantages to the analyses in this study. *N. gonorrhoeae* with nonvariable Opa expression, in an Opa-deleted background (Opaless), allowed for exact control of which Opa protein was expressed on *N. gonorrhoeae*. Using predominantly expressing Opa$^+$ *N. gonorrhoeae* in a background with limited Opa variation capacity (Δ*opaBEGK*) is more similar to bacterial phase variation dynamics *in vivo*, allowing us to assess the role of Opa phase variability and selection for Opa phenotypes in the context of infection. For *N. gonorrhoeae* expressing Opa proteins that bind both CEACAM1 and -3, the ability to phase-vary enhanced survival of the Opa phase-OFF bacteria in the population after exposure to neutrophils, increasing the recovery of *N. gonorrhoeae* in the infection mix over time. Thus, the ability of *N. gonorrhoeae* to avoid phagocytic killing by neutrophils is affected by both Opa expression status and the specific Opa protein being expressed.

The two Opa expressors in this study that were most readily phagocytosed and killed by neutrophils, OpaD and OpaI of FA1090, engage multiple CEACAMs, including CEACAM1 and CEACAM3 (23). Neutrophils constitutively express CEACAM3 at a relatively low level, regardless of their activation status, while CEACAM1 expression is upregulated with exposure to cytokines (33). The non-CEACAM binding strains could bind to heparan sulfate proteoglycan (HSPGs) to mediate interaction with neutrophils. Opa proteins are also highly positively charged at neutral pH (pI ~11) and may interact with cell membranes in an ionic but non-receptor-mediated manner. To fully understand the mechanisms by which *N. gonorrhoeae* expressing OpaD and OpaI strongly associates with human neutrophils will require methods for manipulation of receptor expression in these primary, terminally differentiated cells, which are not currently available. It is also possible that in addition to characteristics of the Opa protein itself, the amount of Opa protein stably expressed on the bacterial surface influences bacterial interactions with neutrophils, although this is a less likely explanation for $Opal_{nv}$

and $OpaD_{nv}$, where the nonvariable *opa* allele is under the control of the native *opaD* promoter. Future studies that investigate a broader array of Opa proteins for their receptor-binding and survival profiles after exposure to neutrophils will help to better understand how the *N. gonorrhoeae* Opa protein repertoire contributes to infectivity.

We were surprised to see that formation of mature phagosomes, release of granule content by neutrophils in response to *N. gonorrhoeae*, and, to some extent, release of ROS occurred regardless of which Opa protein was expressed. CEACAM1 is a canonically inhibitory receptor, containing an ITIM motif in its cytosolic domain. Other literature has shown a reduction in cellular activity and proliferation upon CEACAM1 binding (14, 15). In contrast, CEACAM3 contains an ITAM domain and has been shown to be activating (16, 17). Signaling downstream of CEACAMs involves recruitment of kinases leading to $p47^{phox}$ activation and consequent NADPH oxidase assembly (34, 35). In mouse promyelocytes transduced to express human CEACAMs, cross talk between receptors leads to activation of signals downstream of CEACAM3, when the cells are presented with *N. gonorrhoeae* expressing a CEACAM1-only binding Opa protein (36). Interaction of HSPGs, the secondary receptor of Opa proteins, with β-integrins has been shown to activate neutrophils in a similar manner (37). We anticipate that the similarities in phagosome maturation, degranulation, ROS, and death observed in the current study are due to Syk activation and Src recruitment downstream of CEACAM3 activation, precipitated by either CEACAM1 or CEACAM3 binding. Previous work by Sarantis and Gray-Owen showed that neutrophils were activated to a similar extent in response to either CEACAM1 or CEACAM3 being bound by *N. gonorrhoeae* (36). Our work is in agreement in that we demonstrate here that primary granules mobilize and are delivered to both the *N. gonorrhoeae*-containing phagosome as well as the neutrophil membrane. However, our work reports less pronounced differences between the Opa-expressing and Opaless strains than we reported previously (30). While we do not have a direct explanation for this difference, variation in human subjects' neutrophils or other unidentified features of the infection milieu could contribute.

Compared to the more conserved CEACAM1, human CEACAM3 has evolved relatively recently and is specifically expressed on neutrophils and other granulocytes (38). It has been proposed that CEACAM3 expression is an evolutionary tactic by the human innate immune system to attempt to control infection by pathogens that target CEACAM1. In addition to *Neisseria*, multiple pathogens are known to express outer membrane proteins that bind to CEACAM1, including *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Helicobacter pylori*, enabling bacterial colonization and survival (39–41). Some viruses also can utilize CEACAM1 as a cellular receptor (42). Similar to *N. gonorrhoeae*, none of the known species of CEACAM-binding bacteria have an adhesin that binds solely to CEACAM3. Our data and that of others show that *N. gonorrhoeae* with the ability to bind to CEACAM3 is more likely to be phagocytosed and killed by neutrophils (17, 30, 36, 43–48). Together these observations support a model where CEACAM3 plays an important role in controlling infections by human-targeting pathogens, but, in turn, these pathogens take advantage of recombination, mutation, and phase variation to generate an array of related adhesins, some of which evade binding to CEACAM3. This model is supported by the fact that there is a selection for Opa^- bacteria after exposure to human neutrophils when the *N. gonorrhoeae* inoculum is predominantly expressing phase-variable CEACAM1 or CEACAM3 binding OpaI or OpaD proteins, which is not observed in bacterial populations expressing *N. gonorrhoeae* that do not engage CEACAM3.

Opa proteins are adhesins not only for human neutrophils, the focus of this work, but also human epithelial cells. In particular, Opa-CEACAM interactions enable successful infection by allowing binding to the epithelial cells at the site of infection as well as preventing shedding of those epithelial cells to drive longer-term colonization (49–51). In contrast, the enhanced phagocytosis of Opa^+ *N. gonorrhoeae* by neutrophils leads to decreased bacterial survival, as we and others have shown. In addition, the predominance of Opa-expressing *N. gonorrhoeae* varies with the menstrual cycle, which has been attributed in part to sex hormone-based changes in expression of proteases and other innate immune effectors to which Opa^+ *N. gonorrhoeae* is more sensitive (52).

Along these lines, we reported that OpaD$^+$ *N. gonorrhoeae* is more sensitive than Opaless to killing by bactericidal/permeability-increasing protein (30). Hormonal changes may also affect how well *N. gonorrhoeae* survives inside host cells (53).

Given the competing needs of *N. gonorrhoeae* to colonize epithelial surfaces yet avoid clearance by soluble and cellular immune effectors, phase variability of Opa proteins is advantageous to *N. gonorrhoeae* on a population level. In particular, as uncovered in this study, the phagocytic and antimicrobial activities of neutrophils drive selection in the population for *N. gonorrhoeae* that have phase-varied-OFF expression of CEACAM3-binding Opa proteins because of the enhanced phagocytic killing of the CEACAM3 expressors. However, *N. gonorrhoeae* expressing Opa proteins that are less rapidly phagocytosed and killed does not experience the same negative selection. These data, along with the understanding that Opa proteins are important for epithelial binding, suggest that the possession of numerous Opa genes, each independently phase variable and with their own receptor-binding properties, allows the *N. gonorrhoeae* population to constantly test its environment to maximize the ability to colonize while avoiding immune clearance. These results provide one explanation for why human gonorrheal exudates commonly contain Opa$^+$ *N. gonorrhoeae* when some Opa$^+$ bacteria are more susceptible to phagocytic killing by neutrophils. Since primary Opa sequence does not indicate receptor specificity or selectivity, the dynamics of *opa* gene recombination, mutation, and phase-variable expression are especially important to adapt to different conditions during infection and together enable the overall persistence of *N. gonorrhoeae* in its obligate human hosts.

MATERIALS AND METHODS

Bacteria used in this study. All *N. gonorrhoeae* strains used in this study are in the FA1090 background, constitutively encoding the pilin variant 1-81-S2 due to a mutation in the G4 sequence upstream of *pilE* (22, 54). Opaless (Δ*opaA-K*) and OpaD$_{nv}$ (Opaless with a constitutively expressed, non-phase-variable *opaD* allele in the *opaD* locus) strains were described previously (22). OpaI$_{nv}$ and Opa50$_{nv}$ were created in a manner similar to that of OpaD$_{nv}$, with the non-phase-variable genes placed into the *opaD* locus. Opa50$_{nv}$ was previously described (55). OpaI$_{nv}$ was created by transforming into Opaless a synthesized *opaI* with the OpaD$_{nv}$ nonvariable signal sequence, flanked by ~500 bp upstream and downstream of the *opaD* locus (Genewiz). Transformants were selected by their colony opacity and confirmed by sequencing. Opa54$_{nv}$ was created by cloning a constitutively expressed, non-phase-variable version of *opa54* from strain MS11 (gift of S. Gray-Owen, University of Toronto) (56) into the pKH35 complementation plasmid (57) and then incorporating the allele between *lctP* and *aspC* in Opaless by spot transformation and selection using chloramphenicol (0.5 μg/mL) (23). In Opa54$_{nv}$, Opa54 expression is induced by growing *N. gonorrhoeae* in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Δ*opaBEGK* strain, in which the four transparent Opa proteins that do not confer a strongly opaque phenotype on FA1090 were deleted in-frame (*opaB*, *opaE*, *opaG*, and *opaK*), was previously described (22). Predominantly OpaA$^+$, OpaF$^+$, and OpaI$^+$ expressors in the Δ*opaBEGK* strain were selected by eye by their colony opacity. For Opa phase-variable bacteria, expression of the single Opa protein of interest was confirmed by Western blotting bacterial lysates with a panel of FA1090 Opa-specific antibodies (a gift from M. Hobbs, University of North Carolina) (58). Western blotting was similarly used to assess the Opa$^-$ predominance of the Δ*opaBEGK* population. The phase-variable *opaA*, *opaF*, and *opaI* sequences were extrapolated from the FA1090 genome sequence using the genomic locations previously reported (22). *opaD* (22), *opa50* (55), and *opa54* (23) sequences were published previously and confirmed after introduction into Opaless by DNA sequencing.

Bacterial growth conditions. *N. gonorrhoeae* were grown overnight on gonococcal medium base (GCB; Difco) plus Kellogg's supplements (59) at 37°C with 5% CO_2. *N. gonorrhoeae* was grown in rich liquid medium (GCBL) with Kellogg's supplements overnight with rotation at 30°C and then back diluted twice and grown with rotation at 37°C as previously described (28). Piliated *N. gonorrhoeae* was enriched at the final dilution by collecting naturally sedimented bacteria for transfer into fresh medium. Opa54$_{nv}$ was grown in the presence of 1 mM IPTG under all liquid conditions.

CEACAM binding of Opa$^\pm$ *N. gonorrhoeae* using imaging flow cytometry. Glutathione *S*-transferase (GST)-tagged N-terminal domains of human CEACAM1 (N-CEACAM1) and CEACAM3 (N-CEACAM3) were purified as in reference 23. Opa$^+$ *N. gonorrhoeae* or the Δ*opaBEGK* parent bacteria (1×10^8 CFU/mL) was incubated with GST-tagged N-CEACAM (N-CEACAM1 and N-CEACAM3) for 30 min at 37°C with end-over-end rotation. *N. gonorrhoeae* incubated without any N-CEACAM was used as a negative control. *N. gonorrhoeae* was then washed and stained to detect the presence of CEACAM with anti-GST antibody, as previously described (23). Bacteria were then resuspended in 2% paraformaldehyde with 5 μg/mL 4′,6-diamidino-2-phenylindole (DAPI). Bacteria were processed using the ImageStream X Mk II imaging flow cytometer and analyzed with INSPIRE and IDEAS v. 6.2 software packages (Amnis Luminex Corporation). Cells were gated by singlets, focused cells, DAPI expression, and AF488 expression (23).

The binding profiles of $Opa50_{nv}$, $Opa54_{nv}$, and $OpaD_{nv}$ were previously reported (23) as a percentage of the bacterial population positive for GST. For each sample, at least 40,000 cells were analyzed.

Neutrophil isolation. Venous blood was collected from healthy human subjects in accordance with a protocol approved by the University of Virginia Institutional Review Board for Health Sciences Research (protocol number 13909). Neutrophils were isolated via dextran sedimentation followed by a Ficoll gradient as previously described (28). Neutrophils were resuspended in Dulbecco's phosphate-buffered saline (DPBS; without calcium and magnesium; Thermo Scientific) containing 0.1% dextrose and used within 2 h of isolation.

Neutrophil ROS production. Neutrophils (2×10^5) were resuspended in Morse's defined medium (MDM [60]) in the presence of 20 μM luminol. *N. gonorrhoeae* was added at an MOI of 100. Luminol-dependent chemiluminescence was measured every 3 min for 1 h on a VICTOR3 Wallac luminometer (Perkin-Elmer) as previously described (34). One representative of $\geq$3 biological replicates is presented. Uninfected, untreated neutrophils were used as a negative control in each experiment (34).

Imaging flow cytometric analysis of bacterial association with and internalization by neutrophils. *N. gonorrhoeae* was labeled with Tag-IT Violet proliferation and cell tracking dye (TIV) (BioLegend) in PBS with 5 mM $MgSO_4$ for 15 min at 37°C. Bacteria were then added to neutrophils at an MOI of 1 that were adhered to plastic coverslips in 6-well plates. Neutrophils were suspended in RPMI with 10% fetal bovine serum (FBS) and pretreated with 10 nM human IL-8 (R&D Systems). At the indicated time points, cells were fixed with 4% paraformaldehyde in PBS and removed from the coverslips by gentle scraping as previously described (27). Extracellular bacteria were identified by staining with DyLight 650-conjugated (Thermo Scientific) goat anti-*N. gonorrhoeae* antibody (Biosource), diluted in PBS containing 10% normal goat serum at a final concentration of 1 μg/mL. Cells were then processed on the ImageStream X Mk II imaging flow cytometer and analyzed with INSPIRE and IDEAS v. 6.2 software packages (Luminex Corporation). Gating was completed as previously described (27). Briefly, focused, single cells were gated for low DL650 and then spot counted for TIV^+ *N. gonorrhoeae*. Results are reported as the percentage of the neutrophil population with at least one bacterium that is associated (TIV^+) (bound or internalized).

***N. gonorrhoeae* survival in the presence of primary human neutrophils.** Neutrophils were treated with IL-8 in RPMI 1640 medium with 10% FBS at 37°C with 5% CO_2 and were allowed to adhere to 13-mm-diameter plastic coverslips (Sarstedt) for at least 30 min prior to infection. Mid-logarithmic-phase *N. gonorrhoeae* was exposed to neutrophils at an MOI of 1 and centrifuged together at 12°C to synchronize infection. At the indicated time points, neutrophils were lysed in 1% saponin, lysates were serially diluted and plated, and CFU were enumerated from lysates after overnight growth (28). Results are reported as the CFU enumerated at the indicated time point, divided by the number of CFU associated with neutrophils at time zero min $\times$ 100%.

Determination of intracellular and extracellular bacterial viability. Adherent, IL-8-treated neutrophils were exposed to *N. gonorrhoeae* as for the bacterial survival assays, except *N. gonorrhoeae* was added at MOI of 10. After 60 min, *N. gonorrhoeae* was incubated for 10 min at room temperature in 0.1 M morpholinepropanesulfonic acid, pH 7.2, plus 1 mM $MgCl_2$ containing 5 μg/mL Alexa Fluor 647-coupled soybean agglutinin (Thermo Fisher) to recognize extracellular bacteria. Cells were then permeabilized with 0.1% saponin, and viable and nonviable *N. gonorrhoeae* bacteria were detected using the BacLight LIVE/DEAD viability kit (Invitrogen) as previously described (32).

Neutrophil phagosome maturity. Adherent, IL-8-treated neutrophils were exposed to *N. gonorrhoeae* at an MOI of 1 as described above, except bacteria were first labeled with carboxyfluorescein succinimidyl ester (CFSE) at 1:1,000 in PBS with 5 mM $MgSO_4$ for 25 min at 37°C. After 60 min, cells were fixed and processed for immunofluorescence microscopy as in reference 61. Cells were blocked in PBS with 10% normal goat serum (NGS) for 10 min at room temperature. Extracellular *N. gonorrhoeae* was stained using an anti-*Neisseria gonorrhoeae* antibody, followed by Alexa Fluor 647-coupled goat anti-rabbit IgG (Life Technologies). Cells were then permeabilized in PBS with 10% NGS and 0.2% saponin and stained with an antibody against neutrophil elastase (AHN-10) (Millipore), followed by Alexa Fluor 555-coupled goat anti-mouse IgG (Life Technologies).

Degranulation. Surface expression of primary granule markers was determined as previously described (62). *N. gonorrhoeae* was incubated with primary neutrophils adhered to glass slides for 1 h at 37°C. Cells were then lifted for 10 min on ice using 5 mM EDTA and washed twice in DPBS with 0.1% dextrose. They were then stained with phycoerythrin (PE)-CD63 (BioLegend) for 30 min on ice as an indicator of primary granule exocytosis. Isotype controls (PE-IgG1; BioLegend) were stained using the same protocol. Data were acquired using a Cytek Aurora Borealis spectral flow cytometer and analyzed using FCS Express (De Novo Software). The mean fluorescence intensity of each of the samples was normalized to the Opaless sample as a biological negative control.

Fluorescence microscopy. Images were acquired on a Nikon Eclipse E800 UV-visible fluorescence microscope with Hamamatsu Orca-ER digital camera and analyzed using Nis-Elements (Nikon). At least 5 images were taken for each individual experiment, and $\geq$50 individual bacteria/phagosomes were counted.

Statistics. For all experiments except for the chemiluminescence assay, results are depicted as the means $\pm$ standard errors for $\geq$3 independent experiments (different subjects' neutrophils and different bacterial cultures). Statistics were calculated using GraphPad Prism (version 9.3.1) analysis software. For all experiments, a P value of <0.05 was considered significant. Specific statistical tests are reported for each figure with analysis of variance (ANOVA) used for multiple comparisons for parametric data.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

ACKNOWLEDGMENTS

This work was supported by R01 AI097312 and R21 AI157539 (A.K.C.) and NIH R35 GM131829 (L.C.). A.M.A. and L.M.W. were supported in part by T32 AI007046. L.M.W. was supported in part by the Robert R. Wagner Fellowship at the University of Virginia. M.B.D. was supported by F32 GM136076.

We thank Scott Gray-Owen (University of Toronto) for Opa54-expressing MS11, Marcia Hobbs (University of North Carolina, Chapel Hill) for the gift of anti-FA1090 Opa monoclonal antibodies, Asya Smirnov for assistance with imaging flow cytometry, the UVA Flow Cytometry Core Facility for assistance with spectral flow cytometry, former Criss lab member Louise Ball for creation of the $Opal_{nv}$ strain, and members of the Criss lab for feedback and discussion.

REFERENCES

1. Stern A, Brown M, Nickel P, Meyer TF. 1986. Opacity genes in Neisseria gonorrhoeae: control of phase and antigenic variation. Cell 47:61–71. https://doi.org/10.1016/0092-8674(86)90366-1.
2. Jordan PW, Snyder LAS, Saunders NJ. 2005. Strain-specific differences in Neisseria gonorrhoeae associated with the phase variable gene repertoire. BMC Microbiol 5:21. https://doi.org/10.1186/1471-2180-5-21.
3. Workowski KA, Bachmann LH, Chan PA, Johnston CM, Muzny CA, Park I, Reno H, Zenilman JM, Bolan GA. 2021. Sexually transmitted infections treatment guidelines, 2021. MMWR Recomm Rep 70:1–187. https://doi.org/10.15585/mmwr.rr7004a1.
4. Judson FN. 1990. Gonorrhea. Med Clin North Am 74:1353–1366. https://doi.org/10.1016/s0025-7125(16)30485-0.
5. Criss AK, Genco CA, Gray-Owen SD, Jerse AE, Seifert HS. 2021. Challenges and controversies concerning Neisseria gonorrhoeae-neutrophil interactions in pathogenesis. mBio 12:e00721-21. https://doi.org/10.1128/mBio.00721-21.
6. Palmer A, Criss AK. 2018. Gonococcal defenses against antimicrobial activities of neutrophils. Trends Microbiol 26:1022–1034. https://doi.org/10.1016/j.tim.2018.07.003.
7. Swanson J, Sparks E, Zeligs B, Siam MA, Parrott C. 1974. Studies on gonococcus infection. V. Observations on in vitro interactions of gonococci and human neutrophils. Infect Immun 10:633–644. https://doi.org/10.1128/iai.10.3.633-644.1974.
8. Gray-Owen SD, Dehio C, Haude A, Grunert F, Meyer TF. 1997. CD66 carcinoembryonic antigens mediate interactions between Opa-expressing Neisseria gonorrhoeae and human polymorphonuclear phagocytes. EMBO J 16:3435–3445. https://doi.org/10.1093/emboj/16.12.3435.
9. Bos MP, Kao D, Hogan DM, Grant CCR, Belland RJ. 2002. Carcinoembryonic antigen family receptor recognition by gonococcal Opa proteins requires distinct combinations of hypervariable Opa protein domains. Infect Immun 70:1715–1723. https://doi.org/10.1128/IAI.70.4.1715-1723.2002.
10. Kuespert K, Pils S, Hauck CR. 2006. CEACAMs: their role in physiology and pathophysiology. Curr Opin Cell Biol 18:565–571. https://doi.org/10.1016/j.ceb.2006.08.008.
11. Chen T, Gotschlich EC. 1996. CGM1a antigen of neutrophils, a receptor of gonococcal opacity proteins. Proc Natl Acad Sci U S A 93:14851–14856. https://doi.org/10.1073/pnas.93.25.14851.
12. Virji M, Makepeace K, Ferguson DJ, Watt SM. 1996. Carcinoembryonic antigens (CD66) on epithelial cells and neutrophils are receptors for Opa proteins of pathogenic neisseriae. Mol Microbiol 22:941–950. https://doi.org/10.1046/j.1365-2958.1996.01551.x.
13. Popp A, Dehio C, Grunert F, Meyer TF, Gray-Owen SD. 1999. Molecular analysis of neisserial Opa protein interactions with the CEA family of receptors: identification of determinants contributing to the differential specificities of binding. Cell Microbiol 1:169–181. https://doi.org/10.1046/j.1462-5822.1999.00017.x.
14. Lee HSW, Ostrowski MA, Gray-Owen SD. 2008. CEACAM1 dynamics during neisseria gonorrhoeae suppression of CD4+ T lymphocyte activation. J Immunol 180:6827–6835. https://doi.org/10.4049/jimmunol.180.10.6827.
15. Chen Z, Chen L, Qiao S-W, Nagaishi T, Blumberg RS. 2008. Carcinoembryonic antigen-related cell adhesion molecule 1 inhibits proximal TCR signaling by targeting ZAP-70. J Immunol 180:6085–6093. https://doi.org/10.4049/jimmunol.180.9.6085.
16. Schmitter T, Agerer F, Peterson L, Münzner P, Hauck CR. 2004. Granulocyte CEACAM3 is a phagocytic receptor of the innate immune system that mediates recognition and elimination of human-specific pathogens. J Exp Med 199:35–46. https://doi.org/10.1084/jem.20030204.
17. McCaw SE, Schneider J, Liao EH, Zimmermann W, Gray-Owen SD. 2003. Immunoreceptor tyrosine-based activation motif phosphorylation during engulfment of Neisseria gonorrhoeae by the neutrophil-restricted CEACAM3 (CD66d) receptor. Mol Microbiol 49:623–637. https://doi.org/10.1046/j.1365-2958.2003.03591.x.
18. Adrian J, Bonsignore P, Hammer S, Frickey T, Hauck CR. 2019. Adaptation to host-specific bacterial pathogens drives rapid evolution of a human innate immune receptor. Curr Biol 29:616–630. https://doi.org/10.1016/j.cub.2019.01.058.
19. James JF, Swanson J. 1978. Studies on gonococcus infection. XIII. Occurrence of color/opacity colonial variants in clinical cultures. Infect Immun 19:332–340. https://doi.org/10.1128/iai.19.1.332-340.1978.
20. Jerse AE, Cohen MS, Drown PM, Whicker LG, Isbey SF, Seifert HS, Cannon JG. 1994. Multiple gonococcal opacity proteins are expressed during experimental urethral infection in the male. J Exp Med 179:911–920. https://doi.org/10.1084/jem.179.3.911.
21. Sintsova A, Wong H, MacDonald KS, Kaul R, Virji M, Gray-Owen SD. 2015. Selection for a CEACAM receptor-specific binding phenotype during Neisseria gonorrhoeae infection of the human genital tract. Infect Immun 83:1372–1383. https://doi.org/10.1128/IAI.03123-14.
22. Ball LM, Criss AK. 2013. Constitutively Opa-expressing and Opa-deficient neisseria gonorrhoeae strains differentially stimulate and survive exposure to human neutrophils. J Bacteriol 195:2982–2990. https://doi.org/10.1128/JB.00171-13.
23. Werner LM, Palmer A, Smirnov A, Belcher Dufrisne M, Columbus L, Criss AK. 2020. Imaging flow cytometry analysis of CEACAM binding to Opa-expressing Neisseria gonorrhoeae. Cytometry A 97:1081–1089. https://doi.org/10.1002/cyto.a.24037.
24. Nguyen GT, Green ER, Mecsas J. 2017. Neutrophils to the ROScue: mechanisms of NADPH oxidase activation and bacterial resistance. Front Cell Infect Microbiol 7:373. https://doi.org/10.3389/fcimb.2017.00373.
25. Casey SG, Shafer WM, Spitznagel JK. 1986. Neisseria gonorrhoeae survive intraleukocytic oxygen-independent antimicrobial capacities of anaerobic and aerobic granulocytes in the presence of pyocin lethal for extracellular gonococci. Infect Immun 52:384–389. https://doi.org/10.1128/iai.52.2.384-389.1986.
26. Frangipane JV, Rest RF. 1992. Anaerobic growth of gonococci does not alter their Opa-mediated interactions with human neutrophils. Infect Immun 60:1793–1799. https://doi.org/10.1128/iai.60.5.1793-1799.1992.
27. Smirnov A, Solga MD, Lannigan J, Criss AK. 2020. Using imaging flow cytometry to quantify neutrophil phagocytosis. Methods Mol Biol Clifton NJ 2087:127–140. https://doi.org/10.1007/978-1-0716-0154-9_10.
28. Ragland SA, Criss AK. 2019. Protocols to interrogate the interactions between Neisseria gonorrhoeae and primary human neutrophils. Methods Mol Biol Clifton NJ 1997:319–345. https://doi.org/10.1007/978-1-4939-9496-0_19.
29. Smirnov A, Solga MD, Lannigan J, Criss AK. 2015. An improved method for differentiating cell-bound from internalized particles by imaging flow cytometry. J Immunol Methods 423:60–69. https://doi.org/10.1016/j.jim.2015.04.028.
30. Johnson MB, Ball LM, Daily KP, Martin JN, Columbus L, Criss AK. 2015. Opa^+ Neisseria gonorrhoeae exhibits reduced survival in human neutrophils via Src family kinase-mediated bacterial trafficking into mature

phagolysosomes. Cell Microbiol 17:648–665. https://doi.org/10.1111/cmi.12389.
31. Criss AK, Katz BZ, Seifert HS. 2009. Resistance of Neisseria gonorrhoeae to non-oxidative killing by adherent human polymorphonuclear leucocytes. Cell Microbiol 11:1074–1087. https://doi.org/10.1111/j.1462-5822.2009.01308.x.
32. Johnson MB, Criss AK. 2013. Fluorescence microscopy methods for determining the viability of bacteria in association with mammalian cells. J Vis Exp 79:e50729. https://doi.org/10.3791/50729.
33. Stocks SC, Kerr MA. 1993. Neutrophil NCA-160 (CD66) is the major protein carrier of selectin binding carbohydrate groups Lewisx and Sialyl Lewisx. Biochem Biophys Res Commun 195:478–483. https://doi.org/10.1006/bbrc.1993.2068.
34. Smirnov A, Daily KP, Criss AK. 2014. Assembly of NADPH oxidase in human neutrophils is modulated by the opacity-associated protein expression state of Neisseria gonorrhoeae. Infect Immun 82:1036–1044. https://doi.org/10.1128/IAI.00881-13.
35. Criss AK, Seifert HS. 2008. Neisseria gonorrhoeae suppresses the oxidative burst of human polymorphonuclear leukoctes. Cell Microbiol 10:2257–2270. https://doi.org/10.1111/j.1462-5822.2008.01205.x.
36. Sarantis H, Gray-Owen SD. 2012. Defining the roles of human carcinoembryonic antigen-related cellular adhesion molecules during neutrophil responses to Neisseria gonorrhoeae. Infect Immun 80:345–358. https://doi.org/10.1128/IAI.05702-11.
37. Miller LA, Hong JJ, Kinch MS, Harrison ML, Geahlen RL. 1999. The engagement of beta1 integrins on promonocytic cells promotes phosphorylation of Syk and formation of a protein complex containing Lyn and beta1 integrin. Eur J Immunol 29:1426–1434. https://doi.org/10.1002/(SICI)1521-4141(199905)29:05%3C1426::AID-IMMU1426%3E3.0.CO;2-J.
38. Bonsignore P, Kuiper JWP, Adrian J, Goob G, Hauck CR. 2020. CEACAM3—a prim(at)e invention for opsonin-independent phagocytosis of bacteria. Front Immunol 10:3160. https://doi.org/10.3389/fimmu.2019.03160.
39. Tchoupa AK, Lichtenegger S, Reidl J, Hauck CR. 2015. Outer membrane protein P1 is the CEACAM-binding adhesin of Haemophilus influenzae. Mol Microbiol 98:440–455. https://doi.org/10.1111/mmi.13134.
40. Conners R, Hill DJ, Borodina E, Agnew C, Daniell SJ, Burton NM, Sessions RB, Clarke AR, Catto LE, Lammie D, Wess T, Brady RL, Virji M. 2008. The Moraxella adhesin UspA1 binds to its human CEACAM1 receptor by a deformable trimeric coiled-coil. EMBO J 27:1779–1789. https://doi.org/10.1038/emboj.2008.101.
41. Königer V, Holsten L, Harrison U, Busch B, Loell E, Zhao Q, Bonsor DA, Roth A, Kengmo-Tchoupa A, Smith SI, Mueller S, Sundberg EJ, Zimmermann W, Fischer W, Hauck CR, Haas R. 2017. Helicobacter pylori exploits human CEACAMs via HopQ for adherence and translocation of CagA. Nat Microbiol 2:1–12. https://doi.org/10.1038/nmicrobiol.2016.233.
42. Matsuyama S, Taguchi F. 2002. Communication between S1N330 and a region in S2 of murine coronavirus spike protein is important for virus entry into cells expressing CEACAM1b receptor. Virology 295:160–171. https://doi.org/10.1006/viro.2002.1391.
43. Chen T, Bolland S, Chen I, Parker J, Pantelic M, Grunert F, Zimmermann W. 2001. The CGM1a (CEACAM3/CD66d)-mediated phagocytic pathway of Neisseria gonorrhoeae expressing opacity proteins is also the pathway to cell death. J Biol Chem 276:17413–17419. https://doi.org/10.1074/jbc.M010609200.
44. Sarantis H, Gray-Owen SD. 2007. The specific innate immune receptor CEACAM3 triggers neutrophil bactericidal activities via a Syk kinase-dependent pathway. Cell Microbiol 9:2167–2180. https://doi.org/10.1111/j.1462-5822.2007.00947.x.
45. Sintsova A, Sarantis H, Islam EA, Sun CX, Amin M, Chan CHF, Stanners CP, Glogauer M, Gray-Owen SD. 2014. Global analysis of neutrophil responses to Neisseria gonorrhoeae reveals a self-propagating inflammatory program. PLoS Pathog 10:e1004341. https://doi.org/10.1371/journal.ppat.1004341.
46. Roth A, Mattheis C, Muenzner P, Unemo M, Hauck CR. 2013. Innate recognition by neutrophil granulocytes differs between Neisseria gonorrhoeae strains causing local or disseminating infections. Infect Immun 81:2358–2370. https://doi.org/10.1128/IAI.00128-13.
47. Schmitter T, Pils S, Weibel S, Agerer F, Peterson L, Buntru A, Kopp K, Hauck CR. 2007. Opa proteins of pathogenic neisseriae initiate Src kinase-dependent or lipid raft-mediated uptake via distinct human carcinoembryonic antigen-related cell adhesion molecule isoforms. Infect Immun 75:4116–4126. https://doi.org/10.1128/IAI.01835-06.
48. Booth JW, Telio D, Liao EH, McCaw SE, Matsuo T, Grinstein S, Gray-Owen SD. 2003. Phosphatidylinositol 3-kinases in carcinoembryonic antigen-related cellular adhesion molecule-mediated internalization of Neisseria gonorrhoeae. J Biol Chem 278:14037–14045. https://doi.org/10.1074/jbc.M211879200.
49. Muenzner P, Hauck CR. 2020. Neisseria gonorrhoeae blocks epithelial exfoliation by nitric-oxide-mediated metabolic cross talk to promote colonization in mice. Cell Host Microbe 27:793–808. https://doi.org/10.1016/j.chom.2020.03.010.
50. Yu Q, Wang L-C, Di Benigno S, Gray-Owen SD, Stein DC, Song W. 2019. Neisseria gonorrhoeae infects the heterogeneous epithelia of the human cervix using distinct mechanisms. PLoS Pathog 15:e1008136. https://doi.org/10.1371/journal.ppat.1008136.
51. Muenzner P, Rohde M, Kneitz S, Hauck CR. 2005. CEACAM engagement by human pathogens enhances cell adhesion and counteracts bacteria-induced detachment of epithelial cells. J Cell Biol 170:825–836. https://doi.org/10.1083/jcb.200412151.
52. Cole JG, Fulcher NB, Jerse AE. 2010. Opacity proteins increase Neisseria gonorrhoeae fitness in the female genital tract due to a factor under ovarian control. Infect Immun 78:1629–1641. https://doi.org/10.1128/IAI.00996-09.
53. Edwards JL. 2010. Neisseria gonorrhoeae survival during primary human cervical epithelial cell infection requires nitric oxide and is augmented by progesterone. Infect Immun 78:1202–1213. https://doi.org/10.1128/IAI.01085-09.
54. Cahoon LA, Seifert HS. 2009. An alternative DNA structure is necessary for pilin antigenic variation in Neisseria gonorrhoeae. Science 325:764–767. https://doi.org/10.1126/science.1175653.
55. Martin JN, Ball LM, Solomon TL, Dewald AH, Criss AK, Columbus L. 2016. Neisserial Opa protein-CEACAM interactions: competition for receptors as a means of bacterial invasion and pathogenesis. Biochemistry 55:4286–4294. https://doi.org/10.1021/acs.biochem.6b00124.
56. Kupsch EM, Knepper B, Kuroki T, Heuer I, Meyer TF. 1993. Variable opacity (Opa) outer membrane proteins account for the cell tropisms displayed by Neisseria gonorrhoeae for human leukocytes and epithelial cells. EMBO J 12:641–650. https://doi.org/10.1002/j.1460-2075.1993.tb05697.x.
57. Ramsey ME, Hackett KT, Kotha C, Dillard JP. 2012. New complementation constructs for inducible and constitutive gene expression in Neisseria gonorrhoeae and Neisseria meningitidis. Appl Environ Microbiol 78:3068–3078. https://doi.org/10.1128/AEM.07871-11.
58. Hobbs MM, Malorny B, Prasad P, Morelli G, Kusecek B, Heckels JE, Cannon JG, Achtman M. 1998. Recombinational reassortment among opa genes from ET-37 complex Neisseria meningitidis isolates of diverse geographical origins. Microbiology 144:157–166. https://doi.org/10.1099/00221287-144-1-157.
59. Kellogg DS, Peacock WL, Deacon WE, Brown L, Pirkle DI. 1963. Neisseria gonorrhoeae. I. Virulence genetically linked to clonal variation. J Bacteriol 85:1274–1279. https://doi.org/10.1128/jb.85.6.1274-1279.1963.
60. Morse SA, Bartenstein L. 1980. Purine metabolism in Neisseria gonorrhoeae: the requirement for hypoxanthine. Can J Microbiol 26:13–20. https://doi.org/10.1139/m80-003.
61. Johnson MB, Criss AK. 2013. Neisseria gonorrhoeae phagosomes delay fusion with primary granules to enhance bacterial survival inside human neutrophils. Cell Microbiol 15:1323–1340. https://doi.org/10.1111/cmi.12117.
62. Ragland SA, Schaub RE, Hackett KT, Dillard JP, Criss AK. 2017. Two lytic transglycosylases in Neisseria gonorrhoeae impart resistance to killing by lysozyme and human neutrophils. Cell Microbiol 19:10.1111/cmi.12662. https://doi.org/10.1111/cmi.12662.