Opa protein mediated interactions with human neutrophils affect *Neisseria gonorrhoeae* survival

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Abstract

Neisseria gonorrhoeae (Gc) is the causative agent of the sexually transmitted infection gonorrhea. If left untreated, gonorrhea infections can lead to serious health sequelae including infertility and pelvic inflammatory disease. Gc infection in a human host elicits a potent immune response characterized by a robust recruitment of neutrophils to the site of infection. Despite the immune response, Gc uses a variety of mechanisms to evade killing by neutrophils, allowing for sustained infection, and for viable Gc to be collected from host exudates. One way Gc modulates interactions with human neutrophils is through expression of Opa proteins which interact with CEACAMs. Opa+ Gc are commonly recovered from infected individuals. Previous literature has shown a selection for specific Opa proteins, and that selection seems to be based on their CEACAM binding capability. In this thesis, I sought to determine the capability of Opa proteins to bind to different CEACAMs affects neutrophilic association. I further investigated how those interactions affect neutrophil activation and Gc survival. Understanding these interactions can allow us to better understand the selection for Opa proteins *in vivo.*

Development of a new flow cytometric assay allowed us to determine the CEACAM binding profile of a number of Opa proteins whose binding capabilities had been previously unknown. We were able to characterize the Opa

protein binding of both neutrophilic and epithelial expressed CEACAMs. These data allow us to understand not only how Gc interact with neutrophils, but how they are able to interact with mucosal surfaces in the host.

This thesis utilized a system of primary human neutrophils as well as Gc expressing non-variable Opa proteins in a background in which all the other *opa* genes have been deleted. I also used Gc in an Opa variable background in which the expressed Opas were tested with each use. I found that CEACAM binding affects the ability of Gc to associate with neutrophils, and that increased association with neutrophils was correlated with more bacterial death. Overall, binding to both CEACAM1 and CEACAM3 was found to lead to more neutrophilic association and bacterial death, while binding to only CEACAM1 or to no CEACAMs allowed the bacteria to avoid neutrophils and killing. Finally, I found neutrophilic pressure selects for a Gc population that is either Opa- or expresses Opa proteins that do not bind to CEACAM3. Overall, these data allow us to better understand what Opa proteins are likely to be selected for *in vivo,* and further understand how CEACAM binding by these Opa proteins leads to that selection. In the future, quantifying the expression of Opa on Gc as well as CEACAMs on neutrophils, utilizing an inducible Opa system, and developing a biologically relevant binding assay could begin to answer some of the questions raised by this work.

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Chapter 1: INTRODUCTION

This chapter contains modified portions of the previously published article: Palmer A, Criss AK. Gonococcal Defenses against Antimicrobial Activities of Neutrophils. Trends Microbiol. 2018 Dec;26 (1)

1.1 *NEISSERIA GONORRHOEAE*

Neisseria gonorrhoeae, or the gonococcus (Gc), is a Gram-negative diplococcus that is the causative agent of the sexually transmitted infection gonorrhea. Gonorrhea is transmitted via sexual contact with an infected individual, and there is no reservoir for Gc outside the human body. Over the past several decades, Gc has acquired resistance to essentially every antibiotic commonly used to treat it. The number of Gc infections has also begun to increase over the last few years, with ~1.14 million cases occurring annually in the United States in 2019 (2). These reasons led to the notorious designation of Gc as one of the top three "urgent threats" on the CDC's list of antimicrobial resistant pathogens (2).

1.1.1 Clinical Manifestations

Gc presents commonly as cervicitis in women and as acute urethritis in men, though infection can also occur in the pharynx, the rectal mucosa, or the conjunctiva (3). Rarely, Gc can disseminate into the bloodstream and cause more severe infection in the host, potentially causing arthritis, meningitis, or endocarditis. Men with gonorrhea are more commonly symptomatic than

women, and as such women are more likely to have an untreated Gc infection. With appropriate rapid antibiotic treatment, Gc is cleared from the body with minimal tissue damage. If the infection is left untreated or antibiotics are ineffective, serious sequelae can occur such as pelvic inflammatory disease, an increased risk of ectopic pregnancy, and infertility (4, 5). Further, Gc can be transmitted vertically to a neonate during the birthing process. This mode of transmission leads to Gc being one of the leading causes of bacterial blindness in infants globally (6).

1.2 THE NEUTROPHILIC IMMUNE RESPONSE

Upon Gc infection into a human host, the immune system reacts to Gc infection with an innate immune response characterized by a robust recruitment of neutrophils to the site of infection. Purulent exudates from both men and women with Gc infection are characterized by high numbers of neutrophils (7, 8). Neutrophils, or polymorphonuclear leukocytes, are terminally differentiated cells and are the most abundant leukocytes in human blood. Neutrophils form from multipotent progenitor cells and transition into granulocyte progenitor cells. Exposure to glycoprotein granulocyte-colony stimulating factor (G-CSF) commits the cells to becoming neutrophils (9). As neutrophils are formed, they create three subsets of granules, each containing proteins required for different activity. The first to form while the cells are promyelocytes are primary granules,

followed by secondary granules at the myelocyte stage, and finally tertiary granules at the band cell stage (10). Upon activation, these granules are released in the reverse order from which they were formed in order to assist in cell migration and antimicrobial activity (discussed in section 1.2.3) (11).

Trillions of neutrophils are produced by the body each day (12), where they are released into the blood stream, die within 7-10 hours, and are phagocytosed by macrophages (13, 14). Due to the high number of neutrophils in the body, the release of neutrophils into the blood stream is highly regulated (9). Newly differentiated neutrophils express CXCR4 which binds to CXCL12 , a ligand expressed on the surface of bone marrow stromal cells (10, 15). G-CSF is a key regulator in releasing neutrophils from the bone marrow (16), by interrupting the binding of CXCR4 and CXCL12 (17) as well as reducing the expression of CXCL12 on the surface of bone marrow stromal cells (18, 19). Once in the blood stream, neutrophils are available to respond to injury within the host.

1.2.1 Neutrophil Recruitment to Infection

Mucosal infection with Gc or other microbes, as well as sterile injury, results in the production of chemoattractants, such as interleukin-8 (IL-8), that promote the extravasation of neutrophils from the bloodstream into the infected tissue (20, 21). Neutrophils respond to chemokine gradients by migrating towards the site of infection where they initially interact with endothelial cells via selectins (22).

Microbial structures such as lipopolysaccharide (LPS) induce the expression of selectins on the surface on endothelial cells (23). Selectins are a family of adhesion receptors found on both endothelial cells (P-selectin, E-selectin) (24, 25) and leukocytes (L-selectin) (26). Selectins on endothelial cells interact with leukocytes via the C-type lectin domain at their N-terminus. The interaction between selectins is relatively weak which slows down the neutrophils on the endothelial layer (22, 27). The neutrophils then crawl on the surface of the cells in an intracellular adhesion molecule (ICAM) dependent manner to locate a site to transmigrate through the cells to reach the site of microbial infection (28). Both selectins and ICAMs are upregulated by chemokines including IL-1, TNF-α, or LPS (29–32). Once receptor binding and rolling has slowed neutrophils at the site of infection, IL-8 signaling initiates neutrophil migration to the endothelium, but additional signals including hepoxilin A_3 and leukotriene B_4 drive neutrophils to fully transmigrate and also recruit more neutrophils to the site of infection (33–36). When neutrophils are in inflammatory conditions and are exposed to chemokines such as TNF-α or IFN-γ, they upregulate their phagocytic receptors in preparation for microbial interactions (37–39).

1.2.2 Neutrophil Phagocytosis

Neutrophils are professional phagocytes with the capability to internalize microbes in both opsonic and non-opsonic manners. Antibody opsonization of

the surface expressed components on Gc allows neutrophils to recognize a pathogen and engulf it via either an Fc receptor recognizing IgG or complement receptors (40). Fc receptors expression is upregulated when neutrophils sense chemokines indicating infection (41). Binding to either of these receptors leads to ITAM phosphorylation which initiates activation of Src family kinases, followed by Syk, PI3K, PLC-γ, Rac, and Rho which leads to microbial phagocytosis by neutrophils (42, 43).

Neutrophils can also phagocytose microbes like Gc in a non-opsonic manner. Recognition of opacity associated (Opa) proteins by CEACAM initiates a non-opsonic internalization of the bacteria (44, 45). Discussion of Opa proteins can be found below in section 1.4.2.2 and 1.5. CEACAM3 binding leads to phosphorylation of the ITAM by Src family kinases members which in turn recruits Syk. This activation stimulates the GTPase Rac through Vav, initiating actin reorganization and bacterial entry via a phagosomal cup (46–49).

1.2.2.1 The CEACAM Family of Receptors

The phagocytic CEACAMs are part of a family of 12 glycosylated Ig receptors in which all the receptors have an IgV like N-terminal domain followed by a varied number of immunoglobulin-like constant domains (50). All of the CEACAMs, except CEACAM16, are plasma membrane anchored and face extracellularly, either with a glycosylphosphatidylinositol (GPI) anchor, or with a

transmembrane domain that ends in a cytosolic signaling motif (CEACAMS 1, 3, and 4). Neutrophils express CEACAMs 1, 3, 4, 6, and 8.

CEACAM1 is found on neutrophils as well as epithelial cells, endothelial cells, T cells, B cells natural killer cells, and macrophages. The C terminus of CEACAM1 contains an immunoreceptor tyrosine based inhibitory motif (ITIM) that extends into the cytosol of neutrophils (Figure 1.1). CEACAM1 signaling is characterized by recruitment of tyrosine phosphatases such as SHP-1 (51). SHP-1 recruitment has been shown to lead to downregulation of T cell activation, reducing processes such as ZAP-70 phosphorylation, Th1 and Th2 cytokine production, and cell proliferation (51–53).

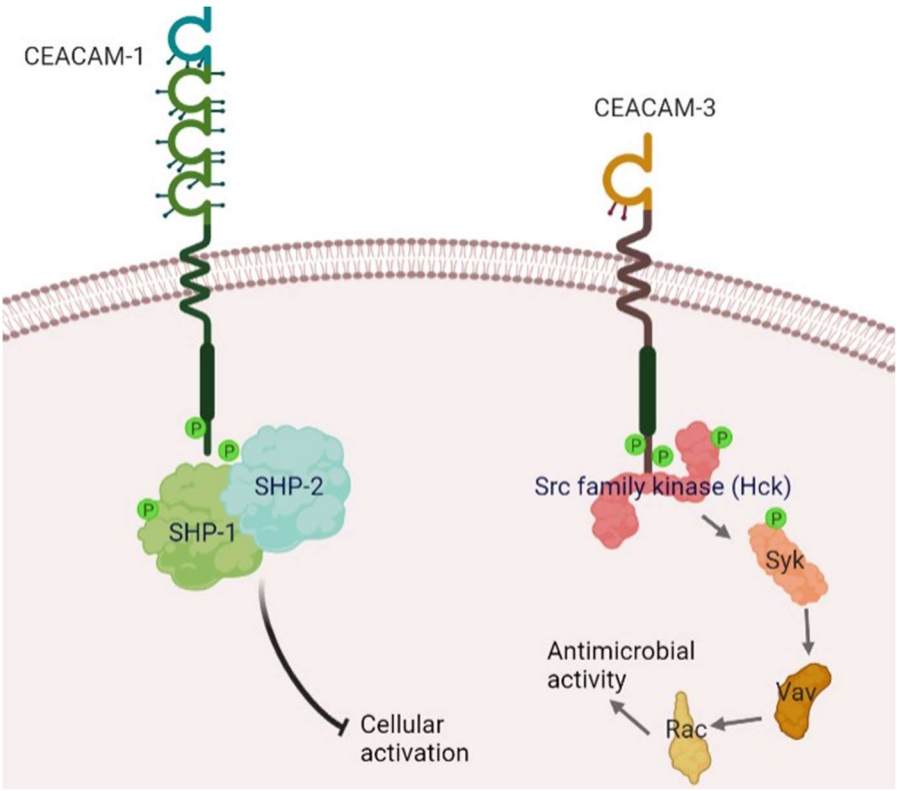

FIGURE 1.1: Signaling downstream of neutrophils CEACAMs 1 and 3

CEACAM1 (green) ITIM phosphorylation leads to recruitment of tyrosine phosphatases SHP-1 and SHP-2 that inhibit cellular activity. CEACAM3 (yellow) ITAM phosphorylation leads to a signaling cascade initiated by recruitment of phosphorylated Src family kinases. This recruitment leads to downstream activation of Vav and Rac. This pathway initiates cellular antimicrobial activity.

CEACAM3 contains an immunotyrosine tyrosine based activation motif (ITAM) and initiates pro-inflammatory signaling cascades in conjunction with TLR signaling through Bcl10 (48, 54, 55). Downstream of CEACAM3, there is recruitment of Syk, Src, Hck and other kinases (55–57), which lead to phagocytosis, granule mobilization, and formation of the NADPH oxidase complex (47, 58). CEACAM3 is unique to granulocytes, implying that it has evolved to promote bacterial killing (59). The Criss lab and others have shown that Opa+ Gc that engage CEACAM3 are readily phagocytosed, induce a potent oxidative burst, and are killed by human neutrophils (58, 60, 61). The proposed signaling of CEACAMs 1 and 3 in neutrophils is shown in Figure 1.1.

Unlike CEACAM1 and CEACAM3, CEACAM6 is glycophosphatidylinositol (GPI) anchored in the membrane of neutrophils. It acts as an adhesion receptor that interacts with CEACAM6 in homotypic interactions as well as heterotypic interactions with CEACAMs 1, 5, and 8 (62, 63). CEACAM6 has also been implicated in cancer signaling (64, 65), and patients with cancer (colorectal, breast, and pancreatic) often see an increased expression of CEACAM6 on their cell surfaces (65, 66). CEACAM6 on neutrophils acts as an adhesin to endothelial cells (62).

1.2.3 Granule Mobilization

Neutrophils contain three subsets of granules. Granule mobilization occurs upon increase of intracellular Ca^{2+} or initiation of signaling of Src kinases (67). Activation causes neutrophil granule components to mobilize to and fuse with the cell membrane to release their components extracellularly, or fuse with a bacteria-containing phagosome and release their contents to expose the pathogen to antimicrobial factors inside the cell (Figure 1.2). Each subset of granules each contains different antimicrobial components (Table 1) (68, 69).

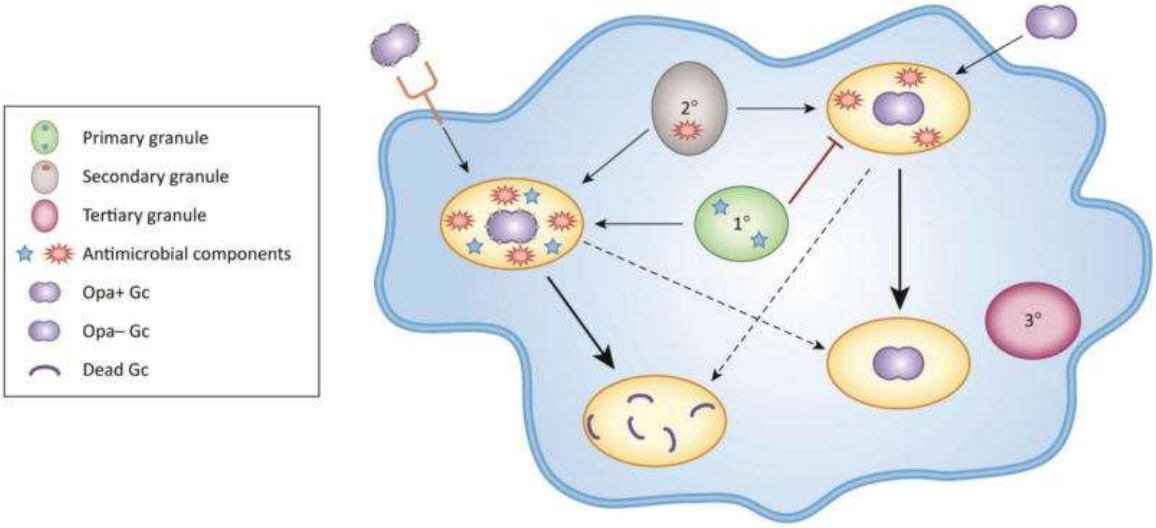

Figure 1.2: Maturation of phagosomes in neutrophils

Left, Opa+ bacteria that interact with CEACAMs are directed into a mature phagolysosome which has fused with both secondary (grey) and primary (green) granules. The majority of Gc that are immediately directed into phagolysosomes are killed. Similar observations are made for IgG-opsonized bacteria that interact with Fcγ receptors (not depicted). Right, in contrast, Opa- bacteria are phagocytosed into a phagosome that is delayed for fusion with primary granules. The majority of Gc in immature phagosomes survive.

Table 1: Selected contents of neutrophil granules

GRANULES	CATEGORY	CONTENTS
Primary	*Antimicrobial proteins*	α-defensins
		Bacteriocidal/Permeability Increasing Protein
		Azurocidin
	ROS production	Myeloperoxidase
	Serine Proteases	Cathepsin G
		Proteinase
		Neutrophil Elastase
	Degradative enzymes	Lysozyme
Secondary	*Antimicrobial proteins*	Lactoferrin
		LL-37
	Degradative enzymes	Collagenase
		Gelatinase
		Lysozyme
	ROS production	Cytochrome b558
Tertiary	*Degradative enzymes*	Gelatinase
		Lysozyme
	ROS production	Cytochrome b558

Tertiary granules are the first to mobilize and contain the least antimicrobial components. They are followed by secondary then primary granules (70). Mobilization is initiated by receptor binding and signaling within the cell, beginning with an increase in intracellular Ca^{2+} (71). Src family kinases also play a major role in granule mobilization, though different kinases in this family affect mobilization of certain subsets of granules: Hck has been shown to be recruited to primary granules while Fgr is recruited to secondary granules (72, 73). Release of granule components to intracellular phagosomes or the extracellular environment is a way for neutrophils to expose both intracellular and extracellular pathogens to potent antimicrobial factors. The antimicrobial activity of primary granules is attributed in part to serine proteases that degrade bacterial membrane proteins as well as process antimicrobial peptides into their mature, active form. Src and Syk kinase activities are implicated in primary granule mobilization to neutrophil phagosomes (47, 56, 57).

1.2.4 Release of Reactive Oxygen Species

In neutrophils, signaling in response to extracellular stimuli leads to the co-assembly of the cytoplasmic and membrane-associated subunits of the NADPH oxidase enzyme, leading to the release of reactive oxygen species (ROS) (74). Formation of the NADPH complex occurs through recruitment of the cytosolic components $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ to the plasma membrane or phagosome

membrane where they associate with gp91^{phox} and p22^{phox} (60, 75). This signaling is mediated via Syk and PI3K (76, 77). Once fully formed, NADPH oxidase converts molecular oxygen to superoxide anion, which dismutates into hydrogen peroxide. The primary granule enzyme myeloperoxidase can then convert hydrogen peroxide into hypochlorous acid, an extremely potent oxidant.

1.3 GC EVADES NEUTROPHIL ANTIMICROBIAL ACTIVITY

Viable Gc are commonly found in exudates from infected individuals (Figure 1.3), despite the gauntlet of antimicrobial activity the human body has developed to kill infecting pathogens.

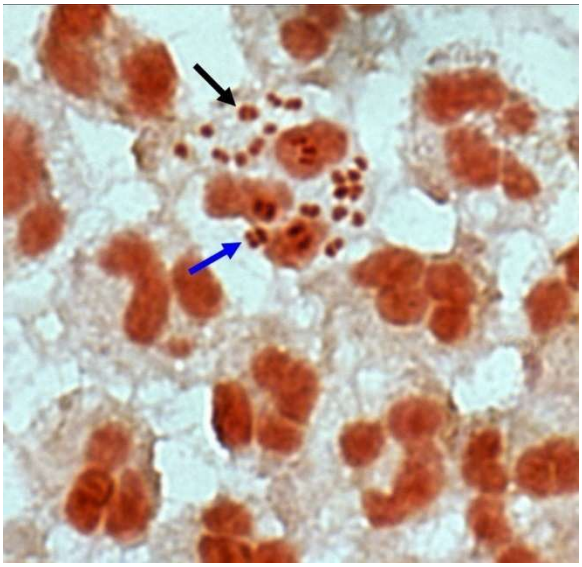

Figure 1.3: Gram stain of a gonorrheal exudate from an individual with uncomplicated urethral gonorrhea. Exudates contain neutrophils with associated Gc, which are found both inside (black arrows) and attached (blue arrows) to the cells.

Pioneering work from Harry Smith and colleagues showed that Gc is phagocytosed by human neutrophils *ex vivo* and *in vivo* and survives inside them (78–80). These observations imply that the neutrophilic immune response to Gc is not effective, despite neutrophils having multiple mechanisms for bacterial killing. These mechanisms include phagocytosis of bacteria, fusion of antimicrobial containing granules to the bacterial phagosome, production of ROS, and release of neutrophil extracellular traps (NETs). Gc uses multiple approaches to enhance its survival in the presence of neutrophils, facilitating bacterial persistence and spread within and between hosts. In this section of my thesis, I will describe the known ways in which Gc evades killing by some of the body's responses to infection.

1.3.1 Nutrient Acquisition Factors

Gc has the ability to scavenge essential metals from the host proteins that are meant to sequester the metals from invading microorganisms (81). This activity is predominantly carried out by transporters that use the energy of the TonB-ExbB-ExbD system to shuttle metals across the outer membrane and transfer them to inner membrane transport systems for delivery to the cytoplasm (82). There are TonB-dependent transporters in Gc that contribute to metal acquisition, including the zinc transporter TdfJ, and TbpAB and LbpAB, which access iron from human transferrin and lactoferrin, respectively (83, 84).

Neutrophils predominantly use glycolysis for ATP production, through which D-lactate is produced (85). Gc encodes two D-lactate dehydrogenases, one cytoplasmic (LdhA) and the other membrane bound (LdhD), as well as an L-lactate dehydrogenase (LldD) (86). LdhA and LdhD contribute to the prolonged survival of Gc inside immune cells. The female genital tract also has high levels of lactate produced by the resident microbiota, suggesting lactate utilization affords Gc an additional metabolic niche *in vivo* (87). Interestingly, exposure to lactate stimulates Gc oxidative metabolism and has been proposed as a mechanism to thwart neutrophil ROS production (88, 89). Thus, utilization of lactate can enhance Gc survival during infection in multiple, non-redundant ways.

1.3.2 Defenses against Cationic Antimicrobial Peptides

During infection, Gc is exposed to two major families of cationic antimicrobial peptides (CAMP), the defensins and the cathelicidin LL-37. These peptides are made by neutrophils and also found in mucosal secretions at sites Gc infects in the human body. A peptide derived from the serine protease cathepsin G also has antimicrobial activity (90, 91). The MtrCDE efflux pump exports CAMPs as well as antibiotics and toxic dyes from the Gc cytoplasm and is crucial for Gc survival in the mouse genital tract (92). Gc detects increasing levels of CAMPs using the MisRS two-component system. The MisR response regulator controls

expression of 94 target genes, 59% of which are involved in envelope integrity of Gc, implying that the MisR response to high levels of CAMPs is to decrease the ability of these antimicrobial peptides to enter Gc (93).

1.3.3 Evasion of Neutrophil Extracellular Traps

First described in a seminal paper by the Zychlinsky group in 2004, NETs are structures composed of DNA that is covered in histones and other CAMPs that are released from neutrophils (94). Canonical NET release via phorbol esters requires ROS production and a cell death pathway termed NETosis. However, NET release without cell death has also been reported (95). Infection with Gc induces NET release from human neutrophils under conditions where the oxidative burst does not occur, suggesting an alternative pathway of NET formation that remains to be elucidated (96). NETs kill microbes in two ways: by increasing the local concentration of CAMPs and by sequestering essential nutrients through proteins like calprotectin (97, 98). Gc has evolved ways to overcome both these antimicrobial strategies. Lipooligosaccharide phosphoethanolamine transferase A (LptA) (see section 1.3.6) is critical for survival of Gc in the presence of NETs, likely due to susceptibility of *lptA* mutant Gc to CAMPs contained within NETs. Gc uses the TonB-dependent transporter TdfH to extract zinc from the abundant neutrophil protein calprotectin in order to survive in NETs (83). Interestingly, TdfH is part of the MisR regulon in Gc

strain FA19, suggesting a connection between cationic antimicrobial peptide (CAMP) sensing and nutrient acquisition (Figure 1.4) (93).

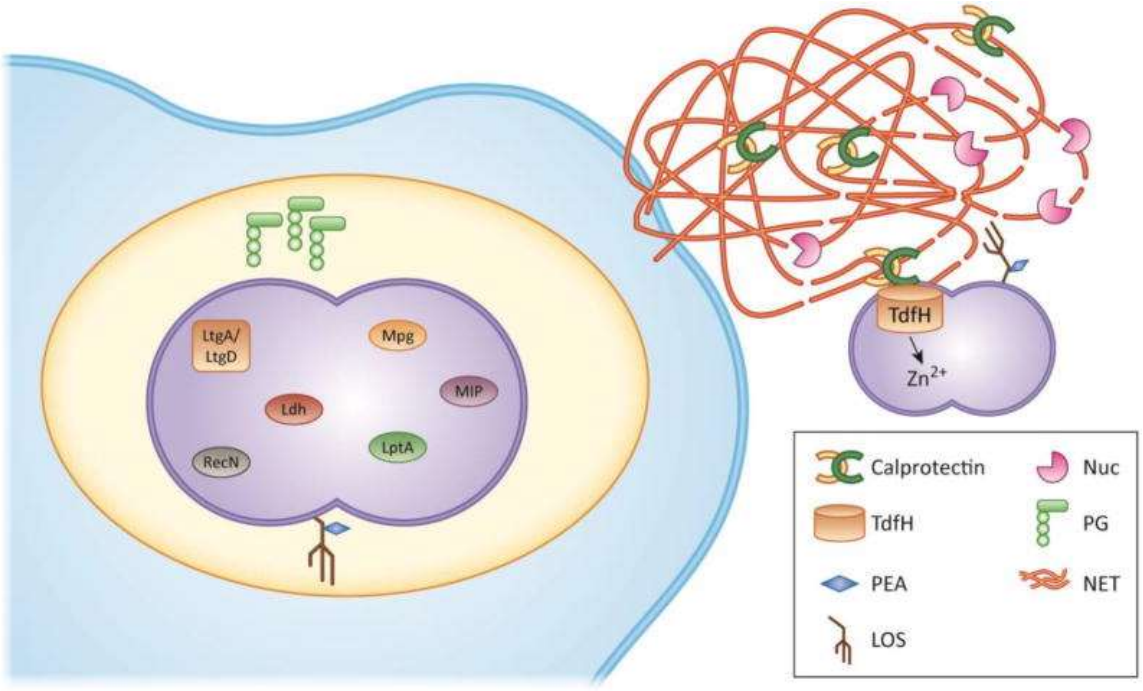

Figure 1.4: Defenses of Gc against neutrophil antimicrobial components

Gc (purple displococcus) both inside the Gc containing phagosome (yellow) and outside of the neutrophil in close apposition with NETs (red lines). Depicted here are selected bacterial defenses that contribute to survival from intracellular antimicrobial components as well as NETs including the metalloprotease Mpg, the LOS-modifying enzyme LptA, the peptidoglycan lytic transglycosylases LtgA and LtgD, the bacterial nucleoid-like protein RecN, and the peptidyl-prolyl isomerase MIP.

Further, studies from the Criss lab found that Gc releases Nuc, a thermonuclease that degrades the NET DNA backbone (Figure 1.4) (99). Gc in which the *nuc* gene was deleted were more susceptible to killing by NETs compared with wild-type Gc. Survival of Nuc deficient Gc was restored upon addition of DNase, purified Nuc, and bacterial supernatants from Nuc-expressing bacteria (99). Nuc is present in all Gc strains examined to date, suggesting conserved roles in Gc biology; in fact, Nuc is also important for remodeling of the DNA-based scaffold of Gc biofilms to facilitate efficient bacterial colonization (100, 101).

1.3.4 Gc Evasion of Phagocytosis

Gc employs mechanisms to limit complement deposition on its surface, thus evading complement-mediated opsonophagocytosis. In particular, porin B, the most abundantly expressed outer membrane protein found on Gc, binds to C4 binding protein and factor H, which limit the classical and alternative pathways of complement activation, respectively (Figure 1.5) (102).

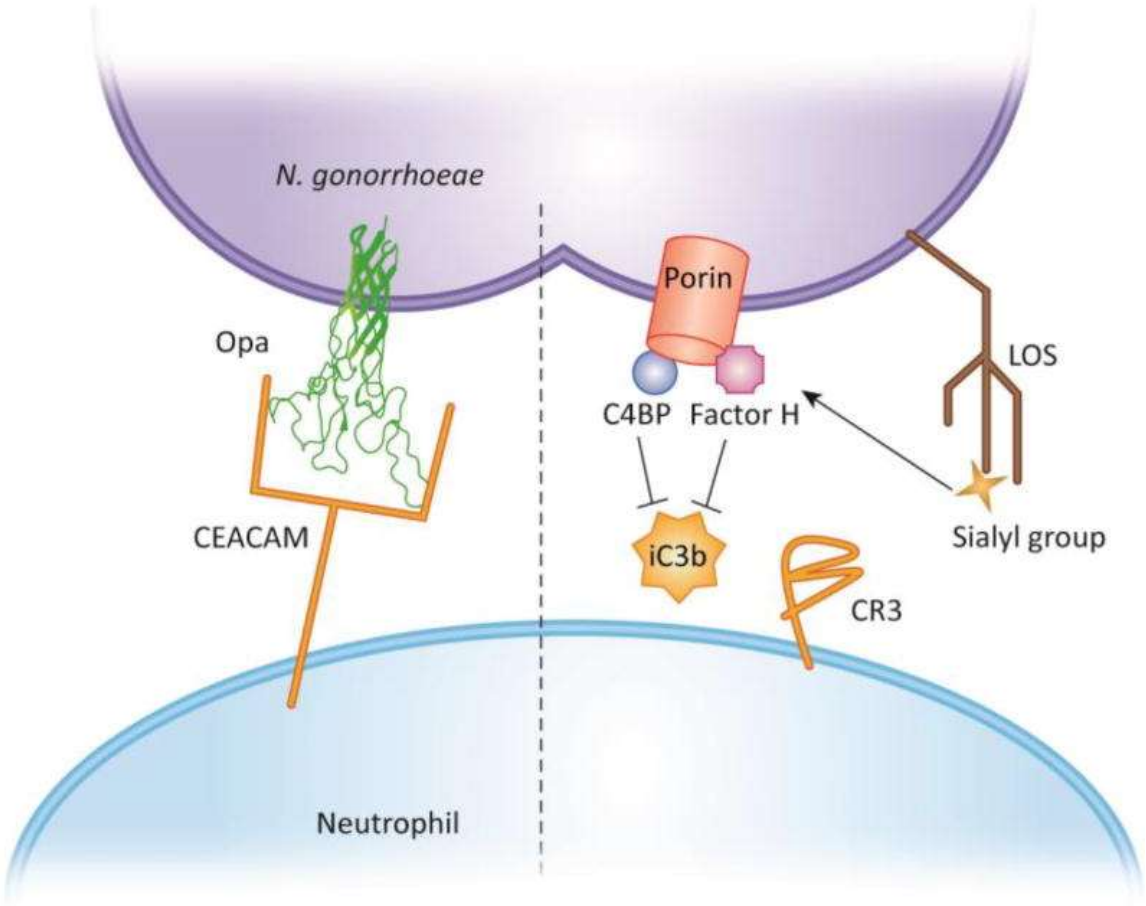

Figure 1.5: Phagocytosis of Gc by neutrophils

Left: Gc Opa proteins bind to neutrophil CEACAMs, which drive nonopsonic phagocytosis of Gc. Right: Factor H and C4 binding protein (C4BP) binding to Gc porin prevents the binding of iC3b and subsequent phagocytosis via the CR3 integrin heterodimer. Sialylation of LOS enhances the deposition of Factor H on porin.

Sialylation of the lacto-*N*-tetraose moiety on the α chain of LOS enhances factor H deposition on porin, explaining in part the increased resistance of sialylated Gc to phagocytosis (103). Whether opsonic or non-opsonic, Gc avoidance of phagocytosis may be enhanced by phase-variable expression of type IV pili, which facilitate Gc attachment to the uropod of migrating neutrophils to escape phagocytosis at the cells' leading edge (104). Together, these findings indicate a subset of Gc in a population has reduced phagocytic interactions with human neutrophils, implying those bacteria will have a relative survival advantage.

1.3.5 Suppression of Reactive Oxygen Species Production

Gc manipulates delivery of primary granule contents into its phagosome, to limit myeloperoxidase release. Engagement of CEACAM by Opa proteins stimulates a potent oxidative burst in neutrophils, which is directed particularly into the phagosome containing Gc (56, 60). In contrast, neutrophils exposed to Opa- Gc do not generate ROS (105). Specifically, the cytosolic NADPH oxidase subunits p40, p47, and p67 were not recruited to phagosomal or plasma membranes, where the membrane-associated subunits gp91 and p22 were found (60). This was correlated with an absence of p47 phosphorylation, a prerequisite to assembly of the holoenzyme. Moreover, Opa- Gc inhibits the oxidative burst in human neutrophils exposed to stimuli such as formylated peptides and *Staphylococcus aureus* (58, 105). The mechanism behind the suppression remains

to be elucidated, but it requires live, metabolically active Gc. *Neisserial* porin has been reported to translocate into the mitochondria of neutrophils and thereby inhibit the oxidative burst (106). Because porin is essential in Gc, these studies used purified porin that was added to human cells. In contrast, a recent study using a porin mutant from the related pathogen *Neisseria meningitidis* found no role for porin in inhibiting the oxidative burst in human neutrophils (107, 108). Thus, the features by which Gc manipulates neutrophil oxidative functions remain enigmatic. Moreover, the role of ROS in the anti-gonococcal activity of neutrophils is questionable due to a variety of Gc defenses against antioxidants (109).

1.3.5.1 Antioxidant Defenses

As an aerobic bacterium, Gc expresses numerous defenses against ROS, including catalase, superoxide dismutase, glutathione peroxidases, a manganese import system, oxidative DNA damage repair enzymes, and methionine sulfoxide reductase (110–113). Gc upregulates numerous genes following exposure to hydrogen peroxide (113). Two of these genes, *ngo1686* and *recN*, confer resistance to both ROS and killing by neutrophils. *ngo1686* encodes a zinc metalloprotease, later named Mpg, which processes the peptide stems of peptidoglycan (114). Mpg expression facilitates full surface presentation of the type IV pilus, a major bacterial adhesin, which was then found to defend Gc

from hydrogen peroxide and killing by neutrophils (114). However, the sensitivity of the *ngo1686* mutant to neutrophils was not restored using neutrophils that cannot make ROS. In fact, Gc mutants in *ngo1686* and the type IV pilus were also more sensitive to LL-37. *recN* also conferred resistance to hydrogen peroxide and neutrophils, but susceptibility of a *recN* mutant to neutrophils was ROS-independent. These findings suggest that Gc senses oxidative stress and responds by upregulating gene products that can defend against a variety of host immune components, including neutrophils themselves. However, they also emphasize that "correlation is not causation", and mechanisms of resistance to individual antimicrobial components may not necessarily be phenocopied in the context of infection.

1.3.6 Manipulation of Phagosome Maturation

Downstream of phagocytosis, Gc limits neutrophil phagosome maturation in order to enable bacterial intracellular survival. In neutrophils, the nascent phagosome matures by fusion with cytoplasmic granules (115). The Criss lab showed that Opa- Gc and Gc that are serum (complement)-opsonized are internalized into phagosomes that lack primary granule components (i.e., immature phagosome) (116). Thus, the bacteria are not exposed to these degradative components early in infection. Residence within an immature phagosome correlates with enhanced bacterial viability, and treating neutrophils

with lysophosphatidylcholine to promote primary granule fusion with the Gc phagosome decreases viability of Opa- bacteria (58). Although the Gc phagosome eventually fuses with primary granules, live bacteria are recovered from neutrophils at these later times. Gc may exploit a delay in phagosome maturation in order to upregulate its antimicrobial defenses as an adaptation to the potentially toxic environment inside neutrophils, or more generally in its human host. Further, Gc modifies LOS by addition of phosphoethanolamine to the 4' phosphate on lipid A, catalyzed by LptA (117, 118). The Criss lab found that *lptA* mutant Gc is more likely to reside in primary granule-positive phagolysosomes, an observation linked with a significant reduction in bacterial survival compared with parent Gc (117). Importantly, Opa- bacteria that are heat-killed prior to exposure to neutrophils also reside in immature phagosomes, indicating that manipulation of phagosome maturation does not rely on active Gc processes (60). This is in keeping with the observation that Gc does not encode secretion systems that inject proteins to manipulate host cell functions (119, 120). Instead, this finding suggests that components on the surface of Gc that interface with neutrophil phagocytic receptors, such as Opa proteins, may influence phagosome maturation.

1.3.6.1 Role of Peptidoglycan dynamics

Peptidoglycan turnover in Gc is unique from other Gram-negative bacteria in that peptidoglycan fragments are poorly recycled from the periplasm and instead released extracellularly (121). Gc lytic transglycosylases LtgA and LtgD, which are responsible for release of immunomodulatory peptidoglycan monomer fragments, confer protection against killing by neutrophils, and specifically against lysozyme and the serine protease neutrophil elastase, found in primary granules (122). The mechanism of protection is likely an increase in the amount of envelope integrity of Gc. Deletion of these transglycosylases rendered Gc more sensitive to killing by neutrophils and more likely to traffic to more mature phagosomes. Because both LOS and peptidoglycan are recognized by TLR and NLR innate immune receptors expressed on neutrophils, signaling through those receptors may more generally modulate granule mobilization to phagosomes, but this possibility remains to be fully explored.

1.4 GC SURFACE COMPONENT VARIATION

Previously, I have described the ways in which Gc can avoid the neutrophil antimicrobial response. In this section, I will describe how Gc manipulates the immune response to allow for more favorable outcomes for the bacteria. Gc has a remarkable ability to direct the body towards an immune response that is beneficial for the bacterium. Gc induces a Th-17-like immune response through

transforming growth factor-beta (TGF-β) regulated mechanisms, leading to the abundant recruitment of neutrophils to the mucosal sites that Gc infects. Antibodies raised against the reduction-modifiable protein (Rmp) on the Gc surface block the antibacterial activity of other anti-gonococcal antibodies, although the underlying mechanism remains enigmatic (123). Gc stimulates high levels of interleukin-10 that, along with regulatory T cells, suppresses T-cell-mediated immunity (124).

Antigenic and phase variation (described in sections 1.4.1-1.4.2) along with expression of Rmp limit antibody-mediated recognition of Gc and the potential for Fc receptor-mediated opsonophagocytosis (123). However, the Criss lab reported that Gc opsonized with a rabbit polyclonal antibody was avidly phagocytosed and killed by adherent, primary human neutrophils (58). This finding implies that if a vaccine against Gc that stimulates an Rmp-independent antibody response can be developed, opsonophagocytosis by neutrophils and other phagocytes may be critical to its efficacy. In support of this possibility, the Ram and Rice groups have found that neutrophils contributed to clearance of Gc when the bacteria were opsonized with an experimental vaccine candidate, the 2C7 antibody that recognizes the β-chain lactose of Gc LOS (125).

To avoid antibody opsonization, Gc undergoes high-frequency variation of antigenic surface structures important for infection. This includes antigenic

variation of type IV pili as well as phase variation of lipooligosaccharide (LOS) and Opa proteins (126–128). Both forms of variation are imperative to bacterial avoidance of the immune response as they alter the surface expressed structures initially recognized by the immune system. The adaptive immune system can create IgG and IgA against the variable Gc surface components (129–131), but their variability makes it unlikely the bacteria will be recognized upon repeat infection. With each surface structure variation, the immune system must make new antibodies against the new structure.

1.4.1 Antigenic Variation

One of the main structural components of Gc that undergoes antigenic variation are pili. Gc pili are multi-complex proteins, with the major component being the variable *pilE* (132). Pili are expressed on the surface of Gc and are involved in attachment to epithelial and endothelial cells, as well as DNA uptake, resistance to neutrophil killing, and bacterial twitching mobility (133–137). Previous studies have shown that attachment by pili to host cells occurs through interactions with membrane cofactor protein (MCP; CD46), though more recent studies dispute this claim (138–140). Type IV pili undergo antigenic variation via replacement of portions of *pilE* with a one of 10-20 silent *pilS* genes located throughout the genome via homologous recombination (141). This recombination is mediated by *recA* (142). Because of the high amount of surface component variability, in this

thesis we utilized Gc strains in which the bacteria cannot undergo the antigenic variation of pili (105, 143).

1.4.2 Phase Variation

Phase variation is an important type of immune evasion found in many bacterial pathogens (144). It is characterized by the on/off switch of a surface component due to a genetic mechanism and occurs independent of recombination machinery. Phase variation is a useful tool to evade the adaptive immune system as it alters or changes the bacterially expressed surface structures. This makes it difficult for an immune response to successfully recognize a population. Phase variation can occur by either DNA inversion, leading to generation of a different protein (145), or by altering the number of nucleotide repeats in the sequence of a gene to affect either localization, expression, or confirmation of that protein. Many pathogens utilize phase variation to evade the immune system including *Bordetella pertussis (fim3)* (146), *Haemophilus influenzae (hif)* (147), and *Moraxella catterhalis (uspA1)* (148).

1.4.2.1 LOS phase variation

LOS are main non-proteinaceous surface expressed structure on Gc. LOS is comprised of an oligosaccharide anchored into the membrane with Lipid A. LOS has been implicated in stimulation of the immune system via interactions with Toll Like Receptor 4 (TLR4) as well as interaction with host cells for adherence

and invasion (149–151). Some human challenge studies have shown that which of the LOS variants is expressed on the surface of Gc may play a role in how symptomatic the infection is in males (7). LOS undergoes phase variation through slipped strand mispairing of a number of genes contained within the same operon (*lgtACD)* (152). During DNA replication, the DNA polymerase can "slip" off the repeated sequence and restart at the incorrect location. This can add or remove nucleotides from the sequence, changing the frame in which the DNA will be translated (153). Dependent on which of these genes are expressed, the length of the oligosaccharide expressed changes. Only the shorter forms of the oligosaccharide structure can be sialylated while the longer stays unmodified (152).

1.4.2.2 Opa protein phase variation

Gc utilizes phase variability for multiple surface components including Opa proteins. Opa proteins undergo phase variability via slipped strand mispairing that occurs in the coding region of each of the ~11 *opa* genes found in the genome due to pentameric repeats of CTCTT (154). Opa phase variability occurs at an estimated 10^{-3} per cell per generation (155). Expression of these proteins is advantageous to Gc in the context of human infection. Human challenge studies have shown infection with phenotypically non Opa expressing (Opa-) bacteria results in collection of Opa expressing Gc at the end of the study (156). Phase

variation of Gc surface structures influences non-opsonic interactions with neutrophils. In particular, non-opsonic phagocytosis of Gc by human neutrophils is mediated by Opa proteins that interact with CEACAMs (157). The interaction between Opa and CEACAM is important for many of the antimicrobial responses discussed above (phagocytosis, release of ROS, granule mobilization), therefore, understanding how this interaction occurs and the way the immune response is controlled is imperative to understand Gc evasion of the immune system**.**

1.5 OPA PROTEIN ROLE IN AVOIDANCE OF IMMUNE RESPONSE

Gonorrheal exudates contain significant amounts of neutrophils, many of which are associated with bound and intracellular Gc (Figure 1.3), and Gc can be readily cultured from neutrophil-rich exudates (158). Gc collected from patient exudates are not only viable, but they commonly express Opa proteins (156). Despite the overall success of Gc in evading killing by neutrophils in many conditions, not all Gc are successful, and some bacteria are killed. Previous work in the Criss lab has shown that expression of the OpaD protein leads to significant bacterial death in the presence of neutrophils (57), which would suggest Opa protein expression is detrimental to Gc, but other literature has shown that there is a selection for Opa expressing bacteria *in vivo* (156). It is important to understand how Opa proteins affect Gc interactions with

neutrophils and how expressing different Opa proteins play a role in those interactions. In this section, I will discuss the importance of Opa proteins for Gc and their function in infection and avoidance of the immune response.

1.5.1 Expression of Opa proteins

If read in frame, *opa* genes are translated into Opa proteins, which are eight stranded beta barrels that span the outer membrane of Gc. Opa proteins are named due to the opaque phenotype seen when Opa expressing Gc are grown on agar (156). The opacity is caused by differential bacterial aggregation when the bacteria are grown on plates, leading to light refraction giving the colonies an opaque look (159, 160). The aggregation is affected not only by the Opa protein expressed and how they interact, but also how the Opa proteins interact with the LOS expressed (161).

1.5.2 Opa protein extracellular loops

Opa proteins have four loops that extend into the extracellular environment. Loop one is semi-conserved between all Opa proteins. Loops 2-3 contain variable regions, while a portion of the fourth loop is conserved (162). The variable regions of loops two and three confer the ability of Opa proteins to bind to receptors. The features of Opa proteins that dictate receptor specificity are independent of primary sequence and instead result from conformations achieved by their extracellular loops (163) (Figure 1.6).

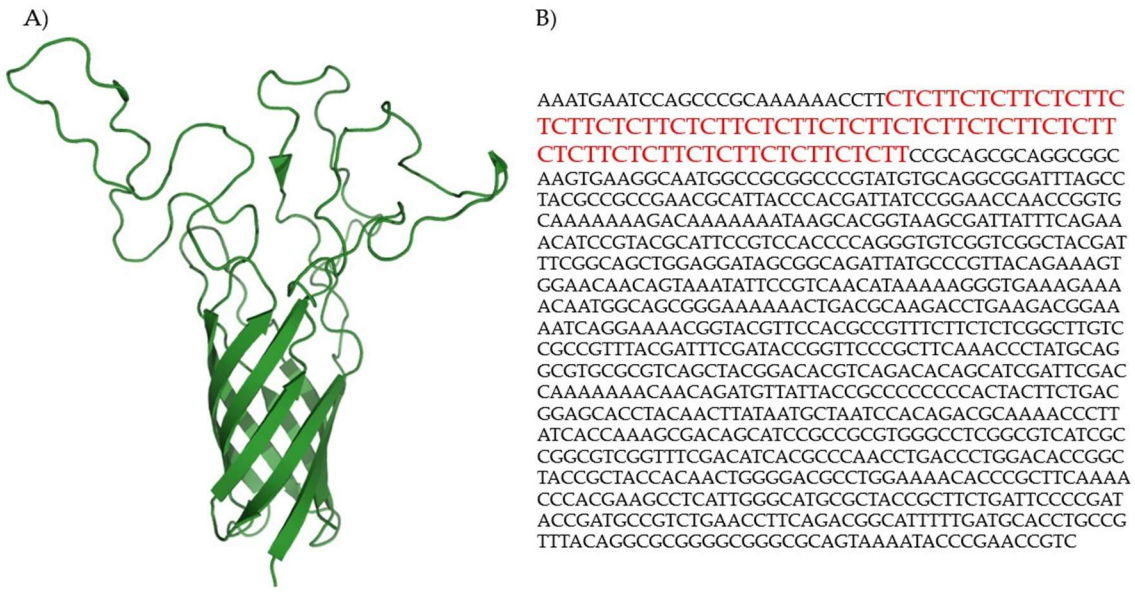

Figure 1.6 Structure and sequence of Opa protein

A) Structure of an Opa protein characterized by the eight stranded beta barrel and the four extracellular loops

B) The nucleotide sequence of the open reading frame and flanking sequence of FA1090 *opaF* (Genbank accession number NC_002946). The 20 CTCTT repeats (referenced in section 1.4.2.2) that lead to the phase variation are shown in red.

Mutating an Opa protein such that individual loops from different Opa proteins with known binding capabilities are placed together to form a new protein changes the binding ability of the Opa (163). This indicates that the interaction of the loops affect the structure of the protein, and change the binding preference of the Opa protein. Due to the variable nature of Opa and the structural requirement for receptor binding, it is not currently known what amino acids are important for receptor binding in Opa proteins.

1.5.3 Opa protein function

The main function of Opa proteins is adhesion to host cells where they are used to confer an intimate attachment to epithelial cells following initial attachment by pili (155, 164). Previous work has shown that Gc interacting with epithelial cells via Opa proteins leads to a higher bacterial load for a longer period. Further, Opa proteins binding to receptors on the surface of epithelial cells prevents epithelial shedding which is one of the main mechanisms by which hosts clear pathogens attached to the epithelium (165). Shedding of infected cells is a common way to clear infection but signaling downstream of the bound receptors inhibits this process, so Gc on the epithelium are able to stay in the host for longer (166). Binding surface receptors confers a close attachment to the epithelial cells, and as the Gc are in anaerobic conditions within a host, they release nitric oxide (NO). Since the Gc are so close to the host cells, the NO is able to penetrate the cells

which leads to upregulation of CD105 on the surface of the epithelial cells. Upregulation of the TGF-β1 receptor CD105 increases integrin activity and leads to more extracellular matrix interactions, which in turn decreases the likelihood of the cells shedding (166). This interaction is shown to be specific to pathogen interactions with CEACAMs, as CEACAM homotypic binding does not confer the same phenotype (165). Without Opa expression leading to the increase in CD105, epithelial cells shed and remove attached bacteria, indicating that Opa expression is important for a sustained infection.

1.5.4 Opa Protein Interaction with CEACAMs

The major receptor for Opa proteins on the surface of human epithelial cells and neutrophils are CEACAMs, though some Opa proteins also have the capability to bind to heparan sulfate proteoglycans (HSPGs) using either vitronectin or fibronectin as a bridge to adhesion (167, 168). Previous studies have shown that Opa proteins have the capability to bind to CEACAMs 1, 3, 5, and 6 (157). As CEACAM1, CEACAM3, and CEACAM6 are expressed on neutrophils and bind Opas, understanding how Opa proteins interact with these receptors and what the outcomes of that interaction are is of interest.

The interaction between Opa proteins and CEACAMs is important for both the bacteria and the immune system. Binding to CEACAMs for Gc can confer a more sustained infection in a host. The immune system hijacks that need of the

bacteria by expressing CEACAMs as a way to attempt to kill pathogens. In fact, epidemiological evidence suggests that the Gc *opa* genes are under evolutionary selective pressure to avoid binding to CEACAM3 while maintaining binding to CEACAM1 and other CEACAMs that assist in epithelial colonization (169).

Activation of CEACAMs by Opa protein binding can lead to a multitude of downstream signaling activities in neutrophils, including phagocytosis, release of ROS, and phagosome maturation as described above (section 1.3). Further, Opa proteins interact with receptors on T cells. CEACAM1 is expressed on T cells and normally inhibits T-cell proliferation and activation (51). When Opa proteins interact with CEACAM1, they downregulate T cell function, inhibiting the ability of the adaptive immune system to have an appropriate response and recognize Gc in the future. These activities may differ dependent upon the way the Opa protein interacts with the CEACAMs.

1.5.5 Selection for Opas *in vivo*

As not all Opa proteins interact similarly with epithelial cells or immune cells, dependent upon which CEACAMs they can bind. As such, not all Opa proteins appear in infection at similar rates. It appears that the Opa proteins on Gc recovered from infection are not random, but what leads to their selection is as of yet unknown. Opa protein expression studied during infection in both female mice and human males found that certain Opa proteins appeared on the

recovered viable Gc more frequently than others, specifically Opa proteins F, I, B, and C of strains FA1090 in humans (170, 171). These proteins were found throughout symptomatic infection in a majority of infected patients. OpaF was commonly recovered especially if OpaF+ Gc were present in the initial inoculum, suggesting that OpaF is important for initial infection as well as a sustained infection. Conversely, OpaI+ Gc appeared later in infection regardless of the presence of OpaI in the initial inoculum (171). Appearing later in infection would suggest an importance in conferring a sustained infection, but less of a role during initial infection of the host.

Sintsova et al. investigated the CEACAM binding capabilities of clinical Gc and observed a selection for Gc expressing Opa proteins that bound to CEACAM1 alone, with a much small proportion of the isolates collected showing binding capability to CEACAM3 (169). These results suggest that the receptor binding of Opa proteins affects how likely they are to successfully infect and sustain infection in a host. Since *opa* genes vary not only within a strain but between strains, many of which have unknown receptor binding specificities, findings from one study can usually not be extrapolated to others.

1.6 DISSERTATION GOALS AND SIGNIFICANCE

An open question in the field drove this thesis work: How does differential CEACAM binding drive the selection for Gc expressing Opa proteins when

confronted with neutrophils during infection? Answering this question will give important insight into the contributions of Opa proteins to Gc pathogenesis, as well as the role of differential CEACAM binding in Gc survival inside a host. Given the overall resistance of Gc to neutrophilic killing despite close contact with the immune cells, my thesis focused on how the phase variable Opa proteins of Gc modulate bacterial/neutrophil interactions. This focus stems from the observation that exudates from infected patients are commonly Opa positive, regardless of Opa expression at time of infection. Prior to this work, our lab published data showing that expression of OpaD on the surface of Gc strain FA1090 led to significant death of the bacteria in the presence of primary human neutrophils (57). However, this work did not take into account that not all Opa proteins interact with neutrophils via the same receptors. Strain FA1090, which I utilize throughout this thesis, was isolated from a patient with disseminated gonococcal infection, and is now a commonly used laboratory strain (172). As Gc undergoes high levels of recombination and variation clinically, the Opas in FA1090 may not be found in clinical isolates, but their CEACAM binding capability allows us to utilize them as a tool.

My main goal was to determine how receptor specificity affected the survival of Gc in the presence of primary human neutrophils. Considering the ability of some Opa proteins to interact with CEACAM1 only while others can bind to

both CEACAMs 1 and 3, and others still interact with CEACAM6, we initially hypothesized that there would be differential survival phenotypes due to signaling downstream of the bound CEACAMs. We hypothesized the ITIM of CEACAM1 would either not activate the more antimicrobial activities of neutrophils (ROS, phagocytosis, granule mobilization) or inhibit those activities via recruitment of tyrosine phosphatases SHP-1 and SHP-2. Further, we hypothesized that activation of the ITAM in the cytosolic motif of CEACAM3 would lead to activation of neutrophil antimicrobial activity. Understanding this interaction would begin to answer the open question in the field; why is there a selection for Opa proteins *in vivo* if activation of CEACAMs on neutrophils via Opa proteins leads to bacterial death? We sought to show that expression of Opa proteins in general is not detrimental to bacteria, but instead certain Opa proteins would lead to bacterial death while others allowed for survival in the presence of a strong immune response. Upon completion of my data collection, we found that there was differential Gc-neutrophil association based on the ability of Gc to interact with varied CEACAMs. Understanding how CEACAM binding of Opa proteins on Gc plays a role in bacterial survival helps us further appreciate how Gc is able to evade the immune response and stay in a human host.

Chapter 2: Imaging flow cytometry analysis of CEACAM binding to Opa-expressing *Neisseria gonorrhoeae*

This chapter is a modified version of the previously published article, Werner LM*, Palmer A*, Smirnov A, Belcher Dufrisne M, Columbus L, Criss AK. Imaging Flow Cytometry Analysis of CEACAM Binding to Opa-Expressing *Neisseria gonorrhoeae*. Cytometry A. 2020 Oct;97(10):1081-1089 (173)

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Lacie Werner – collected and analyzed data, conceived experiments, wrote manuscript

Asya Smirnov – conceived experiments, manuscript editing

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Linda Columbus – conceived experiments, manuscript editing

Alison Criss – conceived experiments, manuscript editing

2.1 INTRODUCTION

Human carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) are a family of 12 receptors with distinct expression patterns on different cell types. Each CEACAM has unique ligand binding capacities, and some CEACAMs have activating or inhibitory cytosolic signaling motifs, while others have no cytosolic tail and contain glycosylphosphatidylinositol (GPI) anchors in the membrane. Thus, the consequence of an interaction between a CEACAM and a potential ligand is dependent on which CEACAM is engaged (174).

CEACAMs are exploited by pathogenic bacteria during infection (166). Examples of pathogens with ligands that bind CEACAMs are *Moraxella catarrhalis* (UspA protein), *Haemophilus influenzae* (OmpP1 protein), *Escherichia coli* (Dr adhesins), and *Neisseria gonorrhoeae* (Opa proteins) (175–177). *N. gonorrhoeae* (Gc) is an obligate human pathogen that causes the sexually transmitted infection gonorrhea. Gc interacts with CEACAMs via outer membrane opacity associated (Opa) proteins. Each strain of Gc encodes 10 or more opa genes, which undergo recombination and mutation to diversify within and among strains (127). Each Opa is phase-variable, such that a single strain can express anywhere from zero to multiple Opa proteins. Gc recovered from infected individuals are predominantly Opa expressors (171). Most Opa proteins in a strain are ligands for one or more human CEACAMs. Receptor binding cannot be predicted from

the Opa primary sequence and is instead dictated by structural characteristics of Opa extracellular loops (178).

CEACAM engages Opa proteins through an extracellular N-terminal immunoglobulin fold (155, 163, 179). CEACAM-Opa binding allows Gc to engage both epithelial cells and neutrophils during infection of the host (155). Characterizing the CEACAM binding profiles of diverse Opa proteins from different strains contributes to our understanding of how Gc interacts with CEACAM-bearing cells to cause productive infection.

Various methods have been used to determine the specificity and selectivity of Opa-CEACAM interactions. Opa binding to CEACAMs that are expressed on the membrane of HeLa cells and CHO cells has been assessed by Western blot, fluorescence microscopy, immunoelectron microscopy, and microtiter plate-based fluorescent detection of bound bacteria (180–185). While mammalian cell lines are a relevant model for host-Gc interactions, the potential for interference by other receptors on the cell surface can confound conclusions about the CEACAM binding capacity of Opa proteins. An alternative approach is to use cell-free purified CEACAMs expressed as fusions to GFP (186–189) or Fc tag (190) that are incubated with Gc and other bacteria expressing Opa proteins, with CEACAM binding assessed by flow cytometry. Previously, our group evaluated Opa-CEACAM interactions by the ability of Gc to bind recombinantly expressed

N-terminus of CEACAM (N-CEACAM). In that assay, the bacteria were incubated with various N-CEACAMs, the supernatant and bacterial pellet were collected and separated by SDS-PAGE, and the N-CEACAM partitioning to the pellet was analyzed by immunoblotting with pan-CEACAM antibody (191). However, this method was subject to variation due to gel loading, blot transfer, and background bands due to non-specific antibody binding, was time-consuming, and reported results for the whole Gc population rather than on a single-bacterium basis.

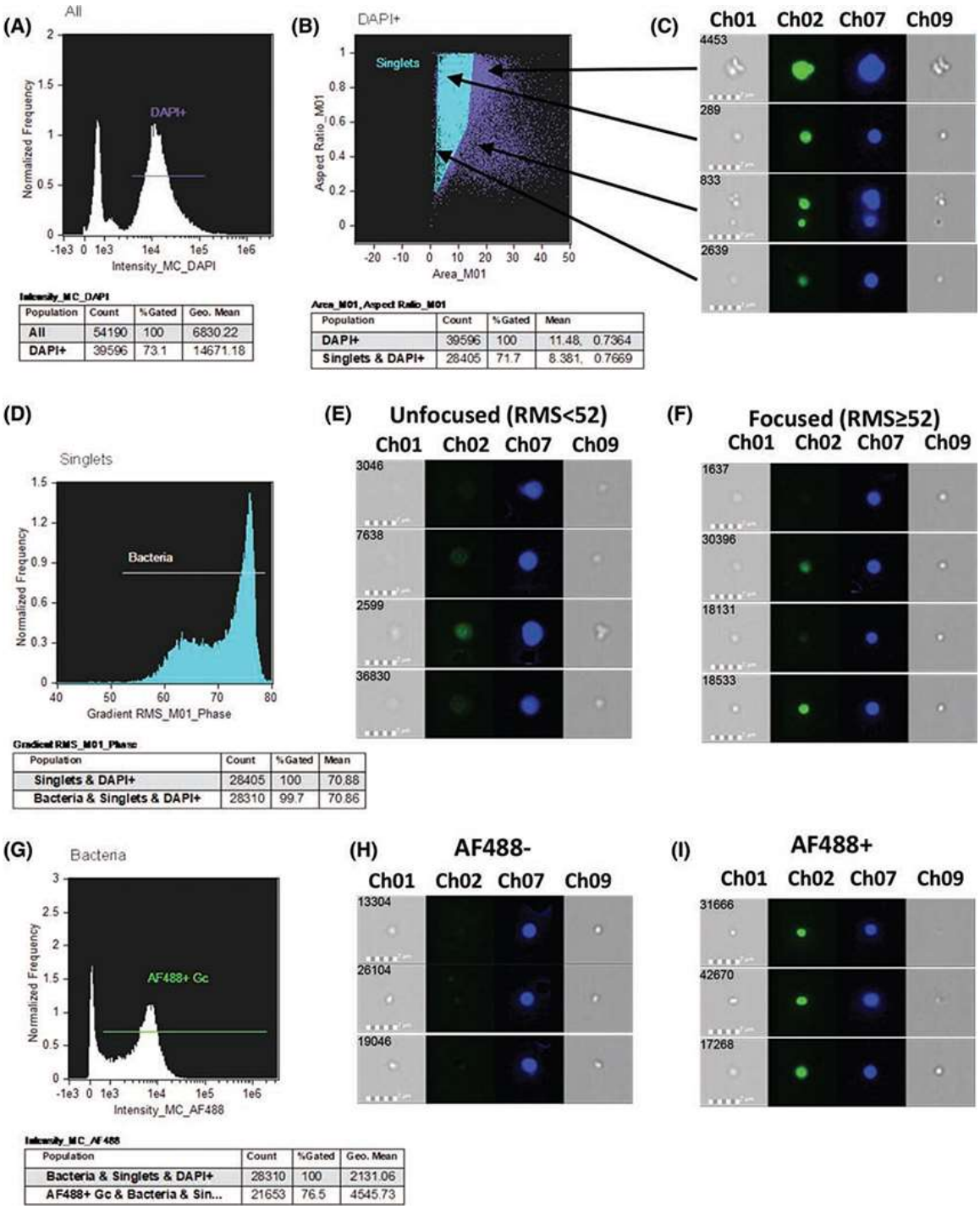
Here we report an approach to define Opa-mediated interactions with recombinant N-CEACAM by using imaging flow cytometry, which offers advantages over conventional flow cytometry. The primary advantage is that given the small size of Gc (0.5 μm diameter as a monococcus and 1 μm as a diplococcus), conventional flow cytometry requires customization and/or extensive calibration and standardization to avoid inaccurate measurements of submicron particles (192–195). In contrast to conventional flow cytometry where single particles are detected bases on a signal threshold, imaging flow cytometry instead identifies objects based on pixel intensities of the particle images that are above background intensity, allowing for accurate detection of bacteria this size. Furthermore, imaging flow cytometry, but not conventional flow cytometry, makes it more straightforward to exclude bacterial aggregates that could skew

the MFI of the whole bacterial population that is analyzed. Theoretically, this approach can be extended to other CEACAM-binding bacteria, and more generally to any ligand-receptor interaction where one of the interacting partners can be made into a soluble fragment.

2.2 RESULTS

2.2.1 Confirmation of a Specific Interaction between Opa and CEACAM by Imaging Flow Cytometry

We developed an imaging flow cytometry-based method to analyze the CEACAM-binding profiles of Gc expressing an Opa protein of interest. In this assay, Gc is incubated with N-CEACAM. A protocol for protein expression and purification of recombinant GST-tagged N-CEACAMs was previously optimized (191). N-CEACAM was recombinantly expressed as a GST-fusion because we previously found the GST moiety prevented N-CEACAM aggregation. The N-CEACAM remaining bound to Gc after washing is detected with an anti-GST antibody, followed by AlexaFluor 488 (AF488)-labeled anti-mouse IgG. Gc was detected with DAPI. The Gc suspension is examined by imaging flow cytometry by gating on DAPI+, focused particles and quantifying AF488 fluorescence in this gate (Figure 2.1).



Population	Count	%Gated	Geo. Mean
All	54190	100	6830.22
DAPI+	39596	73.1	14671.18

Population	Count	%Gated	Mean
DAPI+	39596	100	11.48, 0.7364
Singlets & DAPI+	28405	71.7	8.381, 0.7669

Population	Count	%Gated	Mean
Singlets & DAPI+	28405	100	70.88
Bacteria & Singlets & DAPI+	28310	99.7	70.86

Population	Count	%Gated	Geo. Mean
Bacteria & Singlets & DAPI+	28310	100	2131.06
AF488+ Gc & Bacteria & Sin...	21653	76.5	4545.73

Figure 2.1 **Gating strategy of CEACAM Pulldown**

Gc was incubated with purified recombinant GST-N-CEACAM3 (CCM3), washed, and stained with anti-GST antibody, followed by anti-mouse-AF488 secondary antibody.

Stained bacteria were fixed in PBS containing 2% PFA and 5 μg/ml DAPI. (A) Bacteria were defined as particles with high DAPI intensity. (B) Single bacteria were identified from DAPI+ population. (C) Examples of single and clumped bacteria. (D) Focused bacteria were defined as particles with RMS ≥52. (E) and (F) Examples of bacteria out of focus (E) and focused (F). (G) AF488+ Gc gate includes bacteria with high AF488 fluorescence intensity. (H) and (I) examples of AF488-negative (H) and positive (I) bacteria.

Data are expressed as percent of bacteria that are AF488 positive (Figure 2.2A) and as MFI of AF488 fluorescence (Figure 2.2B) for all particles.

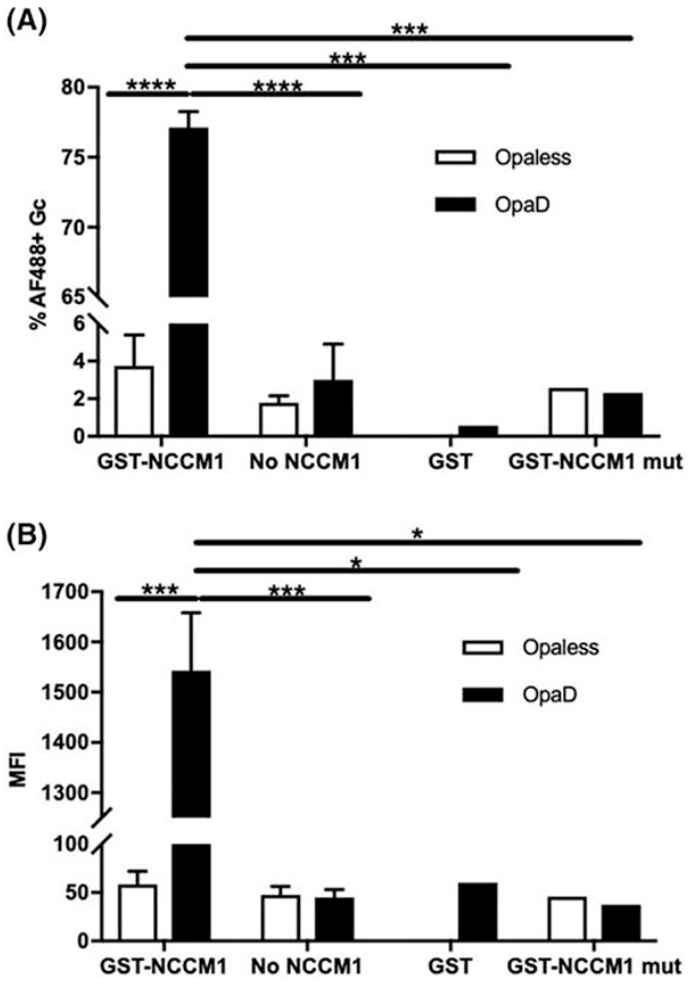


Figure 2.2: Imaging flow cytometry can detect the binding of N-CEACAM1 to Opa-expressing Gc

OpaD+ or Opaless Gc was incubated with GST-tagged N-CEACAM1 (GST-NCCM1), an N-CEACAM1 mutant where the binding interface for Opa proteins was disrupted (GST-NCCM1 mut; point mutations I87A/Q89A/I91A), GST alone, or no protein (No NCCM1). After washing, bacteria were fixed and stained with mouse anti-GST followed by AF488-coupled anti-mouse, along with DAPI. The A) percentage of DAPI+ Gc that are AF488-positive and B) MFI of AF488 for DAPI+ Gc was quantified using imaging flow

cytometry using the gating strategy in Figure 1. Results presented are the mean ± standard error of the mean. n=1-6 experiments. ***, P< 0.001 ****, P< 0.0001 (Student's two-tailed unpaired t test).

The imaging flow cytometry method was first developed using OpaD+ Gc and N-CEACAM1; we previously reported that OpaD binds to CEACAM1 (191) (Figure 2.2). Binding of N-CEACAM1 by OpaD+ Gc was significantly higher by percentage and MFI than binding to Opaless due to the specific interaction of the OpaD protein on Gc and recombinant N-CEACAM1. Several technical and biological controls were employed to validate the specificity of the Opa-CEACAM interaction. 1) In the absence of N-CEACAM1, OpaD+ Gc had minimal AF488+ Gc, i.e. fluorescence from non-specific binding of α-GST and α-MsAF488 to OpaD+ Gc, showing that fluorescence only occurs when CEACAM is present on the measured bacteria. 2) To determine the necessity for Opa expression on Gc to allow CEACAM binding, we showed that for Opaless Gc, percentage AF488+ and fluorescence were not significantly different from the no-CEACAM negative control. 3) The percentage AF488+ and MFI of OpaD+ Gc incubated with recombinant GST was not significantly different from the no-CEACAM negative control, further confirming that the interaction being measured is CEACAM-Opa, not GST-Opa. 4) N-CEACAM1 with three point mutations in the Opa binding interface (CEACAM1-I87A/Q89A/I91A (196)) was no different in interaction with OpaD+ Gc than the non-CEACAM negative control in percentage AF488+ Gc or MFI. Together, these results demonstrate that Gc

specifically binds to CEACAM using Opa as a ligand, and support the use of imaging flow cytometry to examine this interaction.

2.2.2 Titration of N-CEACAMs using OpaD+ Gc

We used the imaging flow cytometry assay to titrate each of several GST-tagged human N-CEACAMs for OpaD+ Gc. The percentage of AF488+ Gc was determined at varying concentrations of each N-CEACAM (Figure 2.3).

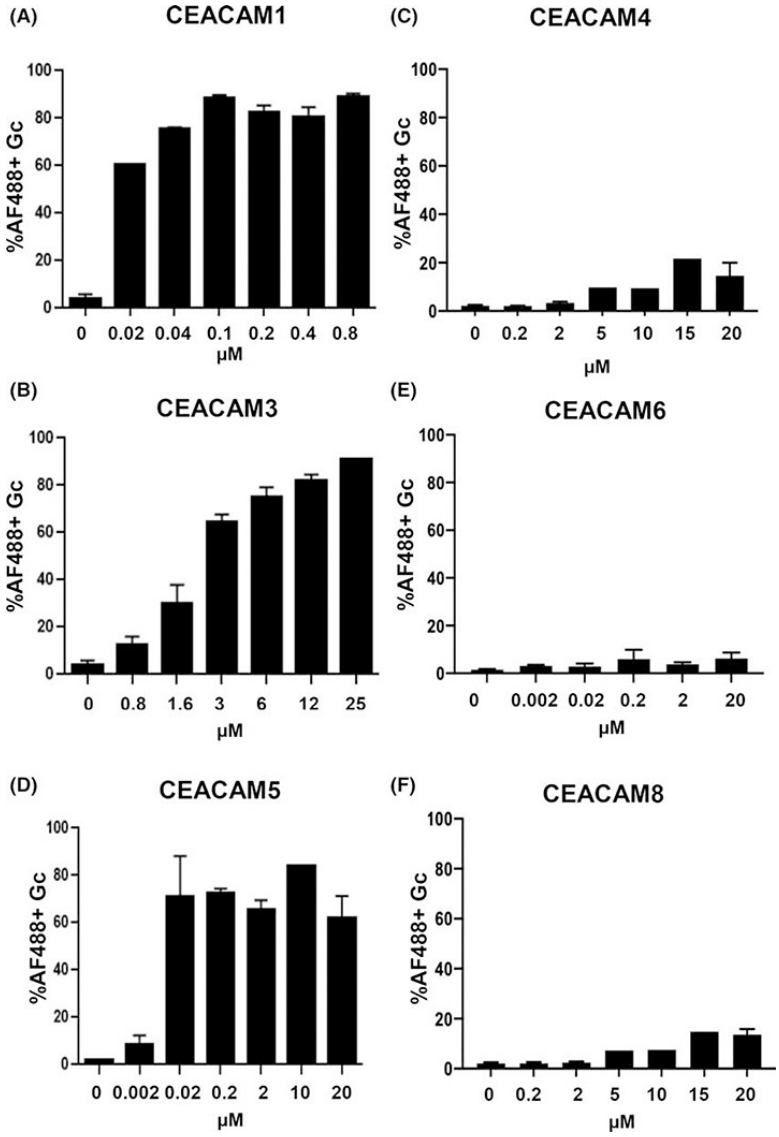


Figure 2.3: Determination of the optimal final working concentration for N-CEACAMs

OpaD+ Gc was incubated with increasing concentrations of each of the recombinant GST-N-CEACAM proteins indicated. Depicted are final concentrations for each protein. Bacteria were processed for imaging flow cytometry as described in Figures 1 and 2. Results are presented as percent of AF488+ bacteria (Similar results were obtained for MFI). Gray diamonds indicate the concentration for each N-CEACAM that was used for

evaluation against the panel of Opa+ bacteria in Figure 4. Results presented are the mean ± standard error of the mean. n=1-6 experiments.

Based on our previous report (191), we predicted OpaD+ Gc would bind to CEACAM1 and 3, and anticipated binding to additional CEACAMs. Opaless Gc was used as a control for non-specific binding at the highest concentration of each N-CEACAM (Figure 2.4). Binding was evaluated as percent of bacteria that were AF488+.

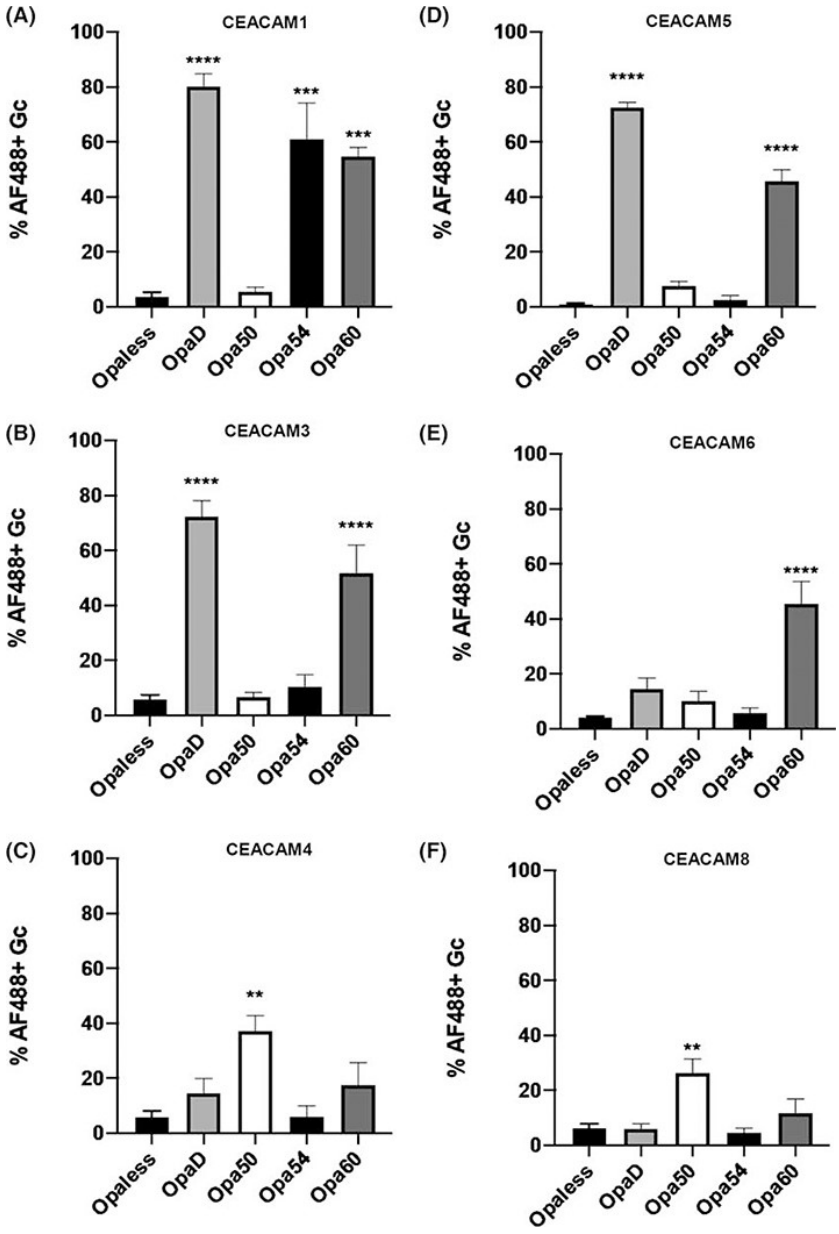


Figure 2.4: CEACAM binding profiles of Opa proteins

The CEACAM binding profiles for Gc expressing OpaD from strain FA1090, Opa50, Opa54, or Opa60 from strain MS11, or no Opa expression (Opaless) were determined by imaging flow cytometry as in Figures 1 and 2, using the final concentration of each GST-N-CEACAM as determined in Figure 3. Each Gc isolate with no added CEACAM served

as a negative control for background AF488+ fluorescence on bacteria and was used as comparison for statistical significance. Results are presented as percent of AF488+ bacteria (Similar results were obtained for MFI). Results presented are the mean ± standard error of the mean. n=2-6 experiments. ***, P< 0.001, ****, P< 0.0001 (1-way ANOVA with post-hoc multiple comparisons and Dunnett's correction).

N-CEACAMs 1 and 5 bound to OpaD+ Gc at low μM concentrations (0.4 μM and 0.2 μM, respectively). In comparison, maximal N-CEACAM3 binding was achieved at 12 μM. CEACAMs 4, 6, and 8 bound poorly to OpaD+ Gc (less than 25%, 10%, and 15% AF488+ Gc, respectively). At these concentrations, no specific binding to Opaless Gc was measured. While these titrations were reproducible for the N-CEACAM preparations used here, each preparation must be optimized for Opa-CEACAM binding based on a similar concentration titration.

2.2.3 CEACAM Binding by each of Four Opa proteins

To characterize the CEACAM binding of other Opas, we selected a concentration at which a CEACAM bound to OpaD. If there was no appreciable binding of OpaD to the N-CEACAM, we selected the highest concentration tested (Figure 2.3). Using these concentrations, the CEACAM binding profiles for Gc expressing OpaD from strain FA1090 or Opa50, Opa54, or Opa60 from strain MS11 were determined by imaging flow cytometry. Opaless Gc incubated with the indicated CEACAM served as a negative control for background AF488+ fluorescence on bacteria, as we have previously shown Opaless Gc does not bind CEACAMs (Figure 2.2) (191). Multiple previous reports have shown that OpaD+ (191), Opa54+ (169), and Opa60+ Gc (163, 185, 191) can bind to CEACAM1, and in agreement, we saw significant binding of CEACAM1 to OpaD+, Opa54+, and Opa60+ Gc (Figure 4.4A). Similarly, OpaD+ (191) and Opa60+ Gc (163, 185, 191)

were reported to bind to CEACAM3, and in agreement, we saw significant binding of CEACAM 3 to OpaD+ and Opa60+ Gc (Figure 2.4B). Previous literature has indicated that no Opas bind to CEACAM4 (197, 198). Using our assay, only Opa50+ Gc bound at significant, albeit low, levels to CEACAM4 (Figure 2.4C). Most CEACAM-binding Opas have been reported to bind CEACAM5 (169), and in agreement, we found that OpaD+ and Opa60+ Gc both bound to CEACAM5 (Fig 2.4D). Additionally, Opa60+ Gc bound significantly to CEACAM6, as previously reported (181) (Figure 2.4E). Finally, no Opas have been reported to bind CEACAM8 (180, 181, 183). Here, only Opa50+ Gc bound CEACAM8 at low levels that were significantly above Opaless background (Figure 2.4F).

The overall consensus between the imaging flow cytometry results and previous publications using other methods supports the use of this assay to rapidly and quantitatively measure the CEACAM binding profile of multiple Opa proteins.

2.3 DISCUSSION

The goal of this project was to create and optimize a straightforward, rapid, specific method for determining bacterial ligand-host receptor interactions by imaging flow cytometry, using Gc Opa proteins and human CEACAMs. Compared with our previous immunoblot-based method (191), imaging flow cytometry allows gating on single, intact bacteria, such that differences within a

population can be evaluated and complications with SDS-PAGE and transfer are avoided. Further, the use of recombinant N-CEACAM in the current approach avoids the complications faced by CEACAM expression in mammalian cell lines, such as efficiency of CEACAM expression, expression of multiple splice variants, and confounding results due to expression of endogenous CEACAM(s). Furthermore, compared to conventional flow cytometry methods, imaging flow cytometry allows single submicron size bacteria to be reliably identified, without customizing and calibrating a conventional flow cytometer, which may not be feasible in a multiuser core facility (192, 199).

We used this approach to measure specific and selective interactions between four different Opa proteins and the N-terminal domains of six different CEACAMs. Gating on single, DAPI+ bacteria ensured that all positive signal detected is from CEACAM bound to intact bacteria, and not from a dimerized protein or protein aggregates that nonspecifically pellet along with the bacteria. When selecting for in focus bacteria, we noted two populations in the gate that have 52<RMS<65 and 66<RMS<78, but since they have the same MFI for AF488 (see Materials and Methods), they do not affect interpretation of results regarding ability of Opa+ bacteria to bind GST-N-CEACAM. At this time we do not know what is responsible for the difference in RMS, but could be due to stage of bacterial growth and/or monococcal vs. diplococcal form. Importantly,

CEACAMs do not bind bacteria that do not express Opa proteins, neither the GST antibody or GST alone bind Opa+ Gc, and mutation of key residues in the CEACAM1-Opa binding interface prevents Opa-Gc interaction (Figure 7). Our results generally correspond to the selectivity of CEACAM-dependent binding of these particular Opa+ Gc reported by us (191) and others (163, 169, 182, 185, 189, 191, 197, 200, 201). Moreover, we found that OpaD+ Gc interacts with CEACAM5, an epithelial CEACAM, which agrees with our observation that OpaD+ Gc binds avidly to epithelial cells (33). Thus, the imaging flow cytometry approach confirms known Opa-CEACAM interactions.

Understanding the selectivity of CEACAM binding to Opa allows us to infer which signaling pathways may be active in the context of host-pathogen interactions. For example, the cytoplasmic tail of CEACAM1 contains an immunoreceptor tyrosine based inhibitory motif (ITIM). ITIM signaling in most cell types relies on activation of SHP-1 and SHP-2 to inhibit cellular activity (53). In neutrophils, this could mean that antimicrobial activity is inhibited by ligand binding to CEACAM1. CEACAM3 is expressed solely on granulocytes like neutrophils. It is thought to be a decoy receptor that pathogenic bacteria bind with the same ligands they use to bind CEACAMs that promote infection (202). Polymorphisms in the CEACAM3 amino acid sequence allow for recognition of a spectrum of bacterial pathogens among human subpopulations (203). CEACAM3

has an ITAM domain in its C-terminus, and this is thought to cause the activation of neutrophils and bacterial killing. Knowing which ligands are able to bind to this specific CEACAM could be important in understanding why some pathogens are killed by granulocytes and others are not.

Previous reports have shown that neither CEACAM4 or CEACAM8 bind to any Opa proteins (197, 198). In this study, low yet statistically significant percentages of Opa50+ Gc were positive for CEACAM4 and for CEACAM8. CEACAM4 is hypothesized to be an "orphan" granulocyte receptor that does not interact with bacterial ligands, although the C-terminal ITAM retains the ability to drive bacterial internalization (198). It is important to note that our system is using non-glycosylated N-CEACAM, which could explain the discrepancy of our results with previous literature. Future studies looking at glycosylated CEACAM4 and CEACAM8 could describe if and how this interaction occurs biologically.

While this method was developed to investigate the binding and selectivity between Opa proteins and CEACAMs, it can be extended to other systems and can be modified for other applications and labeling approaches. Technical variations of this assay could include the use of a fluorophore conjugated to the primary antibody against the recombinant protein of interest or a tagged protein. Further, since we were able to titrate the CEACAM fluorescence based on

concentration of N-CEACAM protein, this assay could be performed as a competition assay where the presence of a competitor will affect the binding of the primary protein of interest, resulting in a loss of signal. Competition assays would help elucidate physiologically relevant questions, such as the affinity of one ligand over another. In this study, we used Opa proteins as the ligand of CEACAM, but there are other types of interactions for which this assay can be used, including other pathogens that are CEACAM-dependent like *M. catarrhalis* (UspA) and *H. influenzae* (OmpP1) (175, 176). More broadly, this system can be adapted to any receptor-ligand pair where one component is presented in its native conformation on the surface of a particle that is of the appropriate size and fluorescence for imaging flow cytometry, and its partner is soluble, in a functional conformation for binding, and can be followed with a fluorescent label. The speed, sensitivity, and throughput of imaging flow cytometry make this an effective approach for analysis of binding to small particles like bacteria.

2.4 MATERIALS AND METHODS

2.4.1 Creation of Recombinant N-CEACAMs 4, 5, 6, and 8

Coding sequences for the recombinant expression of N-CEACAM were synthesized and sub-cloned into the pGEX-2T vector (containing a glutathione S-transferase (GST)-tag) by GenScript. The constructs were designed so that the N-terminal GST tag is separated from the CEACAM domain by a TEV cleavage site

as and a short linker (of the amino acid sequence GGA) as previously reported (191). Plasmids from GenScript were confirmed by Sanger sequencing with forward and reverse primers.

2.4.2 Site Directed Mutagenesis for the Creation of CEACAM1-I87A/Q89A/I91A

Three rounds of PIPE site directed mutagenesis were performed in sequence for the three different mutations introduced. For each round, PIPE-PCR was performed as previously described (204). E. coli Top 10 cells were transformed with the resulting amplicon. DNA was purified using the QiaPrep Spin Miniprep Kit (Qiagen) and mutations were confirmed by Sanger sequencing (Genewiz). The primers for mutagenesis are as follows:

CEACAM1-T87A-F: TTCTACGCACTACAAGTCATAAAGTCAGATCTTGTG

CEACAM1-T87A-R: TTGTAGTGCGTAGAATCCTGTGTCATTCTGGGTGAC

CEACAM1-_89A-F: GCACTAGCAGTCATAAAGTCAGATCTTGTGAATGAA

CEACAM1-_89A-R: TATGACTGCTAGTGCGTAGAATCCTGTGTCATTCTG

CEACAM1-_91A-F: GCAGTCGCAAAGTCAGATCTTGTGAATGAAGAAGCA

CEACAM1-_91A-R: TGACTTTGCGACTGCTAGTGCGTAGAATCCTGTGTC

2.4.3 GST-CEACAM Protein Purification

Recombinant protein was expressed and purified as previously described for the N- terminal domains of CEACAM 1 and 3 (191) using the pGEX-2T vector containing GST-tagged N-terminal domains of CEACAM1, 3, 4, 5, 6, and 8. All proteins were prepared in a final buffer of 20 mM Tris, pH 8.0, 500 mM sodium chloride and 10% glycerol and brought to a final concentration between 4-6 mg/ml using an Amicon Ultra Centrifugal Filter Unit with a 10,000 MWCO (Millipore). Purified GST-CEACAM proteins were stored for at -80◎C. GST-CEACAM protein yields ranged between 0.5-12 mg protein per liter of E. coli culture.

2.4.4 Gc Isolates and Growth Conditions

All Gc isolates are in an FA1090 strain background. Opaless Gc in which all native *opa* genes were deleted was previously described (105). OpaD+ Gc, Opa50+ Gc, and Opa60+ Gc contain non-phase-variable, constitutively expressed versions of each opa gene, cloned into the native *opaD* locus of Opaless Gc (191). An FA1090 Gc strain with a non-phase-variable, IPTG-inducible expressed *opa54* gene was created. *opa54* was amplified out of strain N2027 (strain kindly provided by Scott Gray-Owen, University of Toronto). The *opa54* gene already contained a non-phase-variable signal sequence. Restriction enzyme sites (XbaI and SacI) were added to the *opa54* amplicon for ligation into the Gc

complementation plasmid pKH35 (205) using forward primer AAATTCTAGATCCAAGGAGCCGAA and reverse primer AAAACTCGAGTCAGAAGCGGTAGCG. Both the PCR product and plasmid were digested with XbaI and SacI, ligated together, and transformed into TOP 10 E. coli. Proper insertion of *opa54* into pKH35 was confirmed by restriction digest and DNA sequencing using the primers above (Genewiz). pKH35 with *opa54* was transformed into Opaless Gc and selected for by chloramphenicol resistance at 0.5 μg/mL. Opa54 expression was confirmed by Western blot using the 4B12 anti-Opa antibody after growth of Opa54+ Gc on GCB plates containing 1 mM IPTG. All Gc were grown overnight on GCB plates containing Kellogg's supplements I and II at 37◎ C and 5% CO2, except Opa54+ Gc, where plates additionally contained 1 mM IPTG to induce Opa54 expression.

2.4.5 Opa-CEACAM Pulldown

Protein concentration of N-CEACAMs were measured by absorbance at 280 nm. On the day of the assay, N-CEACAMs were diluted in 500 ◎L to the indicated final concentration per sample in RPMI with 10% FBS (RPMI-10). Gc were collected from GCB plates, normalized to a concentration of 1x108 CFU/mL, washed once in 1x PBS containing 5 mM MgSO4, and resuspended in the N-CEACAM-RPMI-10 solution. Gc and the CEACAM solution were incubated with end over end rotation for 30 minutes at 37◎ C with 5% CO2. After incubation,

bacteria were pelleted at 10,000 x g and washed twice in 1x PBS with 5mM MgSO4.

2.4.6 Bacterial Staining

Gc were resuspended in 75 ◎l 0.5 ◎g/mL mouse anti-GST monoclonal antibody clone p1A12 (Biolegend, San Diego, CA) in RPMI-10 and incubated at 37◎ C for 30 minutes. Bacteria were pelleted at 10,000 g for 3 minutes at room temperature, and the supernatant was discarded. The bacterial pellet was resuspended in 75 ◎L of 5 ◎g/mL goat anti-mouse AF488 (Invitrogen, Carlsbad, CA) in RPMI with 10% FBS and incubated for 30 minutes at 37◎ C. Bacteria were pelleted at 10,000 g for 3 minutes at room temperature, and the supernatant was discarded. Gc were then resuspended in 2% paraformaldehyde (PFA) containing 5 ◎g/mL 4',6-Diamidino-2-phenylindole (DAPI) DNA stain.

2.4.7 Data Acquisition

Samples were analyzed by imaging flow cytometry using ImagestreamX Mk II with INSPIRE® software (Luminex Corporation). Alexa Fluor®488 fluorescence was detected with excitation at 488 nm and emission collected with a 480-560 nm filter. DAPI fluorescence was detected with excitation at 405 nm and emission collected with a 420-505 nm filter. For each sample, a laser LED intensity of 40.59 mW was used for Brightfield 1 and the emission was collected with a 420–480 nm filter; a laser LED intensity 55.00 mW was used for Brightfield 2 and the emission

collected with a 570–595 nm filter. Compensation matrices to remove spectral overlap were calculated for each experiment using DAPI+ bacteria without addition of αGST/αMsAF488, and non-DAPI-labeled OpaD+ Gc bound to N-CEACAM1 with αGST/αMsAF488.

2.4.8 Data Analysis

Data analysis was performed using IDEAS® v6.2 software (Luminex Corporation). The gating strategy for data analysis using this software is outlined in Figure 2.1. A compensation matrix was created from the single-color controls: bacteria singly positive for AF488 or DAPI. This compensation matrix was applied to all files from the experiment. From the DAPI intensity histogram, DAPI+ bacteria were identified (Figure 2.1A). A Scatter plot with Area on the X axis and Aspect ratio on Y axis was generated, and single particles ("singlets") were identified by gating on the cell population with high aspect ratio (>0.2) and low area (<20) (Figure 2.1B and C). A histogram of Brightfield Gradient Root Mean Square (RMS) was generated for single DAPI + bacteria and focused bacteria were identified as a cell population with high (≥52) gradient RMS (Figure 2.1D-F). Within the gate, the population with RMS<65 was not different in MFI for AF488 than the population RMS>65 (4718.38 vs. 4506.08, respectively). A histogram of AF488 intensity for the population of single DAPI+ focused bacteria was created (Figure 2.1G-I). A gate was created in this histogram to

identify the CEACAM+ Gc population. The gating strategy needs to be experimentally determined by each user, by setting the positive gate above the intensity of the cell population in the negative control.

2.4.9 Statistics

Comparisons between single Gc isolates for a given CEACAM were performed using 1-way ANOVA with post-hoc multiple comparisons. Comparisons between fluorescence on OpaD+ Gc after incubation with N-CEACAM1 compared to the addition of no CEACAM or a triple mutant N-CEACAM1 were performed using a student's unpaired t-test. Comparisons between N-CEACAM1 binding to OpaD+ Gc and Opaless Gc were performed using a student's unpaired t-test.

Chapter 3: Variable expression of Opa proteins by *Neisseria gonorrhoeae* **influences bacterial association and phagocytic killing by human neutrophils**

This chapter is a modified version of the previously published article: Alcott AM, Werner LM, Baiocco CM, Belcher Dufrisne M, Columbus L, and Criss AK. Variable expression of Opa proteins by *Neisseria gonorrhoeae* influences bacterial association and phagocytic killing by human neutrophils. J Bacteriol. 2022 Mar 28:e0003522. (206)

CONTRIBUTIONS:

Allison Alcott – experimental design, data collection and analysis, manuscript writing

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Chris Baiocco – data analysis

Meagan Belcher Dufrisne – supplied reagents

Linda Columbus – supplied reagents, experimental design, data interpretation

Alison Criss – experimental design, manuscript writing

3.1 INTRODUCTION

Neisseria gonorrhoeae (Gc) 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 (154, 207); 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 US Centers for Disease Control and Prevention (208). These issues emphasize the need to better understand the mechanisms by which Gc infects and successfully colonizes its obligate human host, in order to develop effective new therapeutics and vaccine targets. Gc infects mucosal surfaces including the nasopharynx, rectum, male urethra, and female cervix. Women are more frequently asymptomatic than men and may not seek treatment (209). Despite a robust neutrophil-rich immune response, Gc that is not cleared from infected tissues leads to tissue damage (210).

Neutrophils (the predominant type of polymorphonuclear leukocyte, PMN) are the first line of defense against many invading pathogens, including Gc. Despite the rapid, robust response of neutrophils to many infectious agents, Gc is able to evade many of neutrophils' effector functions, including neutrophil extracellular traps, ROS release, release of antimicrobial peptides and proteases, phagocytosis

into degradative compartments, and nutritional immunity (described in Chapter 1) (6).

While a subset of Opa proteins have been shown to be able to bind to HSPGs, the major family of Opa-binding receptors are human CEACAMs. CEACAMs are expressed on multiple cell types relevant to *Neisseria*l infection, including neutrophils, endothelial cells, and epithelial cells (10). Human neutrophils express CEACAMs 1, 3, 4, 6, and 8; of these, the *Neisseria*l Opa proteins analyzed to date have been shown to interact with CEACAMs 1, 3, and 6, as well as CEACAM5 on epithelial cells (182, 197, 211, 212). CEACAM3 is thought to have evolved on human and primate neutrophils as a way to combat the myriad microorganisms that exploit CEACAM1 as a colonization factor (203).

Opa protein expression is selected for *in vivo*, with specific Opa proteins appearing more often than others in a given strain background. Most Gc that can be cultured from the secretions of infected individuals express at least one Opa protein (156). In male volunteers that are inoculated urethrally with predominantly phase-OFF Opa-negative Gc, most bacteria that are recovered from symptomatic individuals express one or more Opa proteins (156). 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

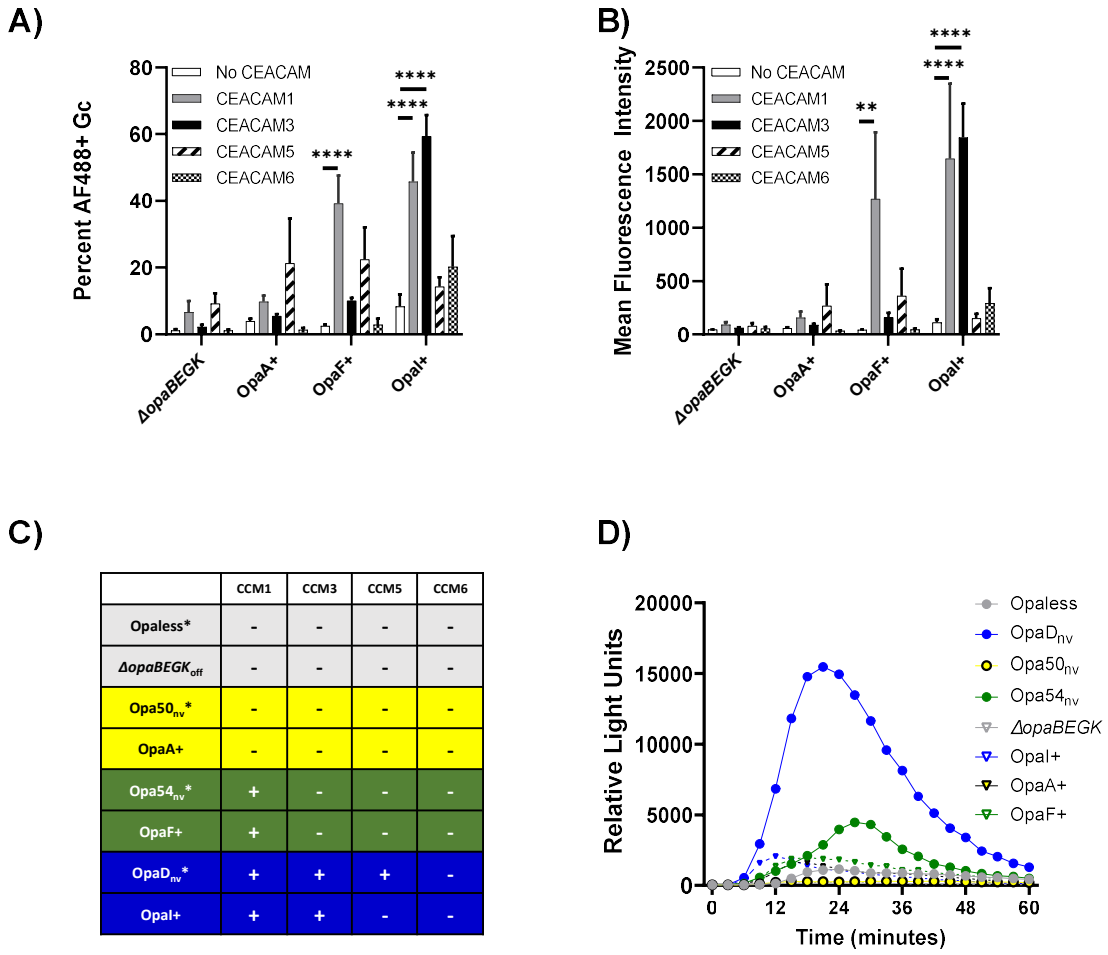
was terminated by antibiotic treatment (171). What drives the selection for expression of particular Opa proteins *in vivo* is not yet clear. However, Sintsova et al. found that primary Gc isolates from urethral and cervical infections more frequently expressed Opa proteins that do not have the ability to bind CEACAM3 (169).

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 Gc when exposed to neutrophils. To this end, we created an isogenic panel of Gc strain FA1090 of different CEACAM-binding profiles, using Opa proteins that are constitutively expressed or are phase varied on 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 Gc resistance to neutrophil killing, where bacteria that do not bind CEACAM3 have a survival advantage.

3.2 RESULTS

3.2.1 Gc Expressing Opa Proteins of Different Receptor-binding Profiles Differentially Activate Human Neutrophils

To investigate Opa-dependent interactions of Gc with neutrophils, we established a panel of Gc expressing single Opa proteins that are predicted to interact differently with human neutrophils (Figure 3.1).



	CCM1	CCM3	CCM5	CCM6
Opaless*	-	-	-	-
ΔopaBEGK$_{off}$	-	-	-	-
Opa50$_{nv}$*	-	-	-	-
OpaA+	-	-	-	-
Opa54$_{nv}$*	+	-	-	-
OpaF+	+	-	-	-
OpaD$_{nv}$*	+	+	+	-
OpaI+	+	+	-	-

Figure 3.1: **Receptor-binding profile of selected Opa proteins and elicitation of the neutrophil oxidative burst**

A, B) Gc predominantly expressing OpaA, OpaF, or OpaI in the *ΔopaBEGK* background, or the Opa- control, was incubated with GST-tagged recombinant N-CEACAM1, N-CEACAM3, N-CEACAM5, N-CEACAM6, or no protein as a control. Binding of CEACAM was recognized with an anti-GST antibody followed by Alexa Fluor 488-coupled goat anti-mouse IgG. The capacity of each Gc strain used in this study to bind each CEACAM was determined by imaging flow cytometry. The percent 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 (A) and (173). Yellow, blue, and green colors are kept consistent throughout the study. Opa+ indicates phase-variable strains, nv indicates non-phase-variable, locked ON strains. D)The indicated strains of Gc at MOI = 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 minutes. Circles denote non-phase variable, "locked" strains of Gc in Opaless; triangles indicate predominantly phase-ON or OFF Gc in the *ΔopaBEGK* background (Opa- bacteria are gray).

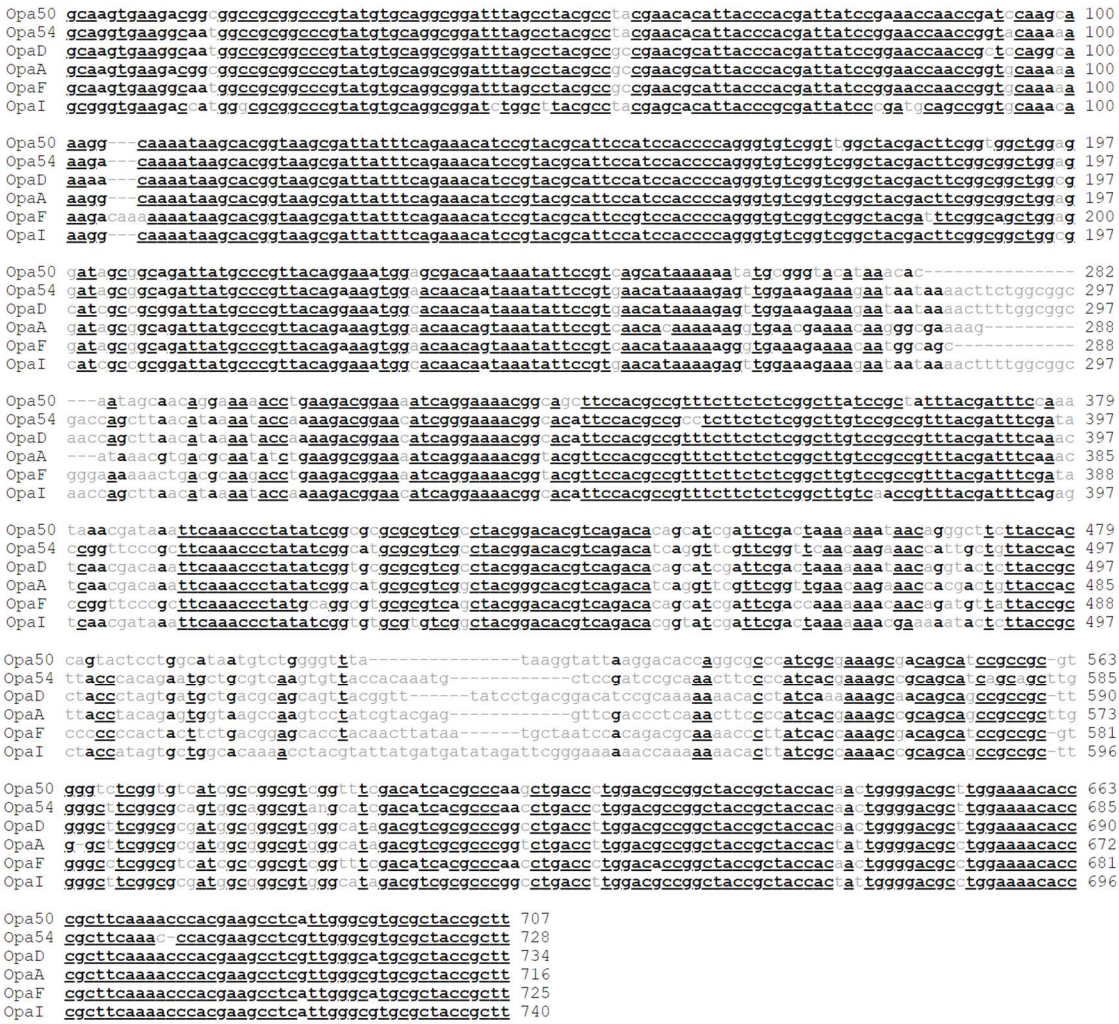
Opa proteins were expressed in two genetic backgrounds, each with its own advantages. In one, constitutively expressed, nonvariable (nv) versions of opa genes were introduced into a piliated derivative of strain FA1090 in which its eleven opa genes were deleted, called Opaless (105). They were either introduced into the opaD locus and driven by the opaD promoter (Opa50nv, OpaDnv), or into an intergenic site between aspC and lctP, under ectopic control of the tac-lac promoter (Opa54nv). The other approach used phase-varied ON Opa expressors (OpaA+, OpaF+, OpaI+) in the piliated FA1090 *ΔopaBEGK* 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 blot using monoclonal antibodies specific to each Opa protein of strain FA1090 (171). We confirmed that each of the used strains grew similarly in media (data not shown), as previously reported by our lab (105).

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 epithelial-restricted CEACAM5, the two other CEACAMs that Opa proteins are reported to bind. Receptor binding was

determined by the ability of Gc expressing defined Opa proteins to precipitate the soluble N-terminal domain of each of these CEACAMs (N-CEACAMs), analyzed by imaging flow cytometry (173). Results are reported as both the percentage of Alexa Fluor 488+ (CEACAM-binding) Gc (Figure 3.1A) and the mean Alexa Fluor 488 fluorescence intensity of the population (Figure 3.1B).

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 (173). 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 (173). These data are consistent with prior reports about Opa54's CEACAM-binding preferences (169, 173) and that Opa50 interacts with heparan sulfate proteoglycans and not CEACAMs (182). Applying the imaging flow cytometry binding assay to the predominantly phase-ON Opa+ Gc, 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 (Figure 3.1A, B). None of the newly tested Opa proteins bound to CEACAM5 or CEACAM6 (Figure 3.1A, B). The nucleotide (Figure 3.2A) and amino acid (Figure 3.2B) sequences of each Opa used in this study are reported.

A


Opa50
Opa54
OpaD
OpaA
OpaF
OpaI

B

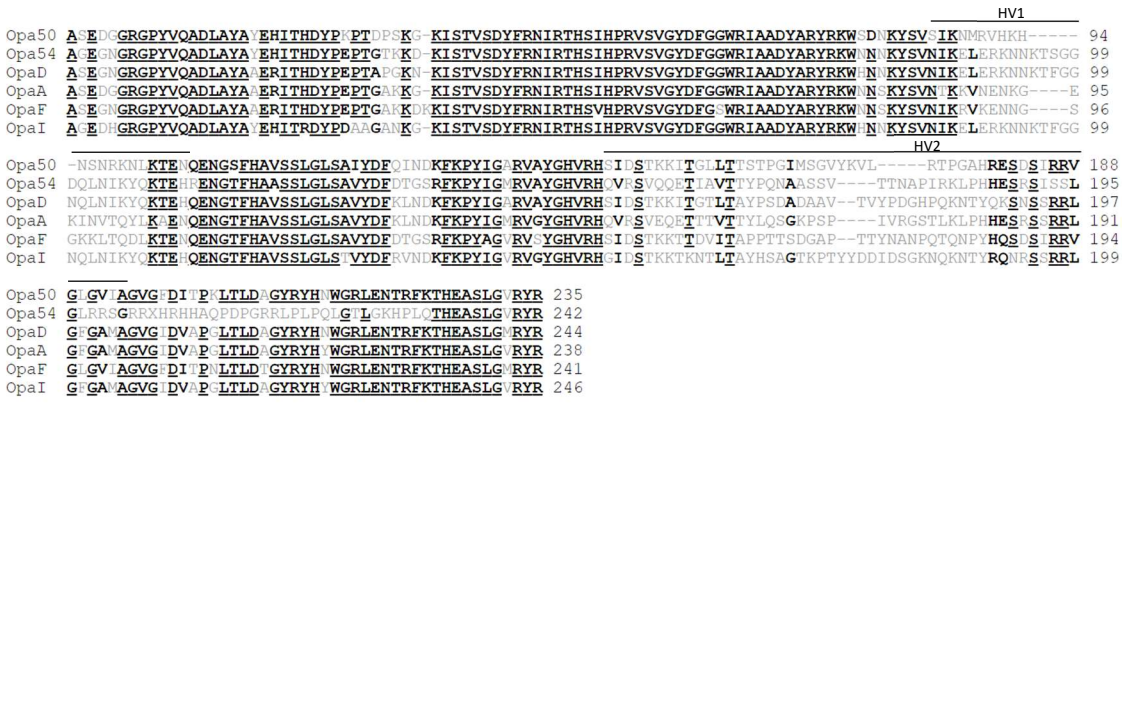

HV1
HV2
Opa50
Opa54
OpaD
OpaA
OpaF
OpaI

Figure 3.2 Opa nucleotide and amino acid sequences used in this study

A) The nucleotide sequence of each of the opa genes in this study, starting with the first codon after the phase-variable signal sequence. Black indicates 100% conservation of the nucleotide across the alignment, grey represents 70% similarity.

B) Amino acid sequence of each of the Opa proteins in this study, starting with the first amino acid of the mature protein (post signal sequence cleavage). Black indicates 100% conservation of the amino acid, grey represents 70% similarity. The hypervariable regions are marked with lines above the amino acids.

Opa50 and Opa54 are from strain MS11; OpaA, OpaD, OpaF, and OpaI from strain FA1090.

The CEACAM binding profiles and genetic background for each strain in this study are presented in Figure 1B. Opaless and ΔopaBEGKoff (*ΔopaBEGK*) are the non-Opa expressing bacteria used throughout this study, which did not interact with any CEACAMs (Figure 3.1A, B) (173) .

To begin to assess how expression of the different Opa proteins affect interaction with primary human neutrophils, we measured neutrophil production of ROS after exposure to equivalent CFU of Gc, 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 (213). ROS does not directly contribute to neutrophil antimicrobial activity against Gc (214, 215), but does reflect the activation state of neutrophils in response to infection. The CEACAM1 and 3 binding OpaDnv and OpaI+ elicited a rapid (within 15 min) ROS response from neutrophils (Figure 3.1D, blue lines). In contrast, the ROS response was slower (peak ~20-30 min) in response to the CEACAM1-only binder Opa54nv. OpaF+ Gc, which also binds CEACAM1 and not CEACAM3, elicited a marginal ROS response from neutrophils, with a peak within 15-20 min of exposure (green lines with triangles). The non-CEACAM binding strains of Gc did not elicit minimal release of ROS from neutrophils, similar to the Opaless and *ΔopaBEGK*

backgrounds (yellow lines; grey 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.

3.2.2 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 developed by our lab, which uses a spot count algorithm to quantify the association and phagocytosis of Gc across tens of thousands of neutrophils per condition (216). These experiments used adherent, IL-8 treated primary human neutrophils as a surrogate for neutrophils that have migrated to sites of mucosal Gc infection (217).

At 15 minutes post-infection at an MOI of 1, fewer than 20% of neutrophils were associated with the non-CEACAM or CEACAM1 only-binding nonvariable Gc (average for Opaless: 4.4%, Opa50nv: 7.7%, Opa54nv: 12%) (Figure 3.3A).

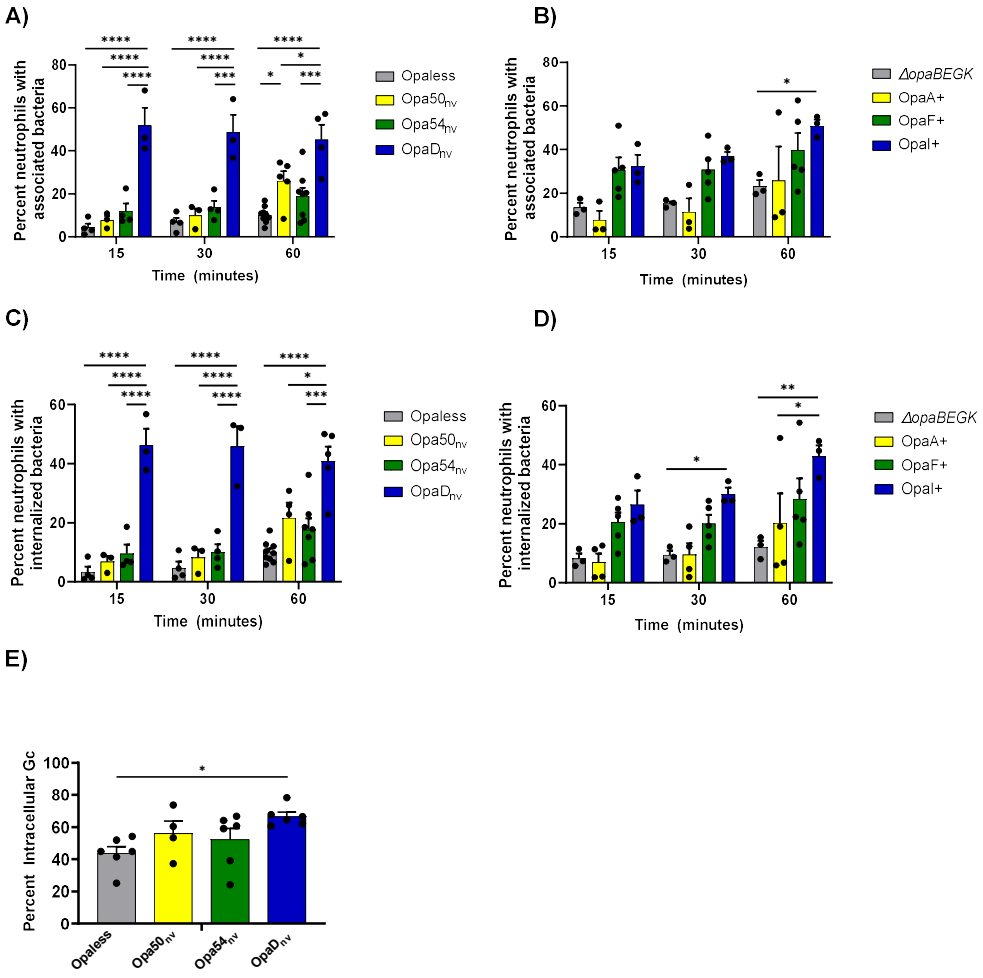


Figure 3.3: Expression of different Opa proteins differentially affects binding and phagocytosis of Gc by primary human neutrophils

The indicated strains of Gc (A, C: constitutively expressed, nonvariable, B, 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-Gc antibody without permeabilization to recognize extracellular bacteria. Neutrophils were analyzed via imaging flow cytometry. A and B report the percent of single, intact neutrophils with ≥ 1 cell-associated bacterium

(TIV+). C and D indicate the percent of neutrophils with ≥ 1 phagocytosed bacterium (TIV+ DL650-). Results are the average of n>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 Gc were labeled with CFSE, then incubated with adherent, IL-8 treated neutrophils. After 60 minutes, cells were fixed and stained. Images were captured by fluorescence microscopy. The percent of intracellular Gc was determined by dividing the number of CFSE+ AF647- (intracellular) Gc by the number of CFSE+ AF647+ (total) Gc. Statistical comparisons were made for n≥4 biological replicates using one-way ANOVA with Tukey's multiple comparisons, with *p<0.05 considered significant.

In contrast, at the same time point, the CEACAM1 and CEACAM3 binding OpaDnv showed significantly more association with neutrophils (OpaDnv: 52%) (Figure 3.3A). Similarly, at 30 minutes and 60 minutes, OpaDnv Gc were significantly more associated with neutrophils than Opaless, Opa50nv, or Opa54nv bacteria (Figure 3.3A). At 60 minutes, significantly more neutrophils were also associated with Opa50nv compared to Opaless.

The phase-varied ON strains followed similar patterns (Figure 3.3B). At 15 and 30 minutes, OpaF+ and OpaI+ Gc showed higher association with neutrophils than OpaA+ or *ΔopaBEGK* bacteria, but these differences were not statistically significant (*ΔopaBEGK*: 14%, OpaA+; 7.7%, OpaF+: 31%, OpaI+: 32%). By 60 minutes, OpaI+ Gc was associated with more neutrophils than the other strains, and significantly increased over *ΔopaBEGK* bacteria (Figure 3.3B). A histogram of the number of Gc counted per neutrophil in each population is presented in Figure 3.4.

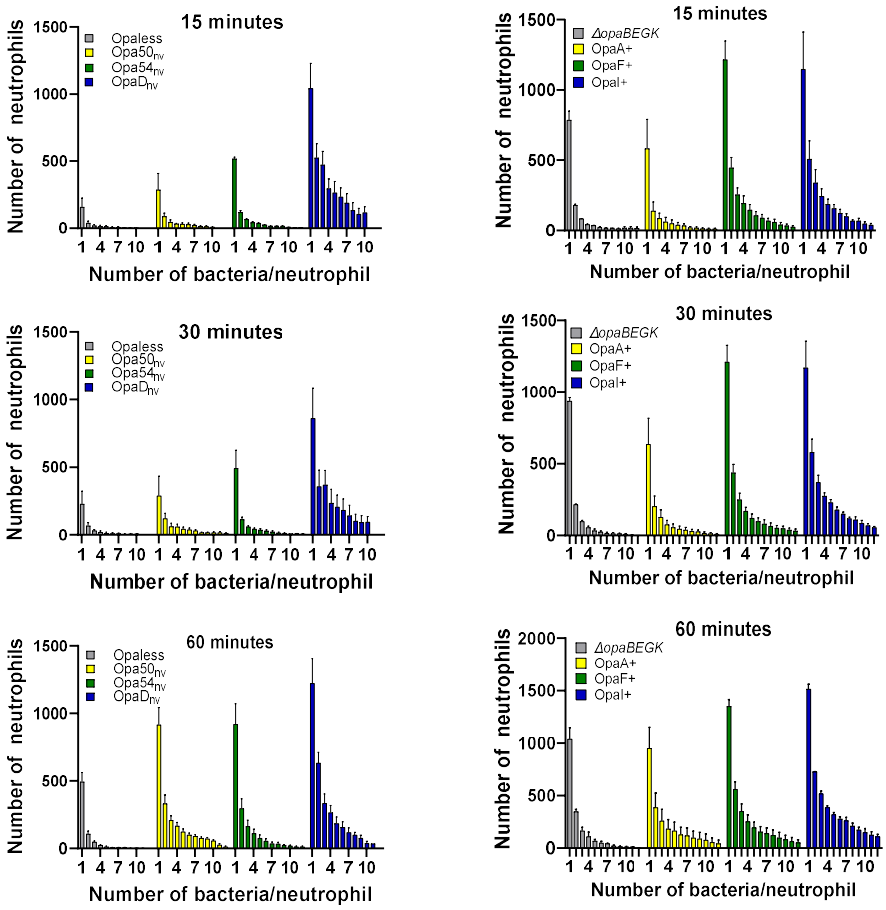


Figure 3.4: Histograms of Gc of different Opa expression states associating with neutrophils over time

Adherent, IL-8 treated neutrophils were exposed to Tag-IT© Violet-labeled locked (A, C, E) or phase-variable (B, D, F) Gc of the indicated Opa profiles. Neutrophils were fixed at 15 (A-B), 30 (C-D), or 60 (E-F) min, stained for extracellular Gc with Dylight 650-labeled anti-Gc antibody, and processed for imaging flow cytometry as in Figure 2. From the data generated with a spot count algorithm in Figure 2, the number of neutrophils with the indicated number of cell-associated Gc was quantified. Neutrophils with zero Gc are

not reported. Data are the average of n≥3 biological replicates. Colors are as in Figure 2.

The same dataset was analyzed for bacterial phagocytosis, with the output being percent of neutrophils with intracellular Gc (see Materials and Methods for details). Significantly more neutrophils contained intracellular OpaDnv Gc compared with the other nonvariable bacteria, at all measured time points post-infection (Figure 3.3C). There were no significant differences between Opaless and Opa50nv or Opa54nv at any of the time points. Similar to the percent association data for the phase-varied ON Gc, neutrophils internalized more OpaI+ than OpaA+ or *ΔopaBEGK* at 30 and 60 minutes post-infection, with the difference between OpaI+ and OpaA+ being statistically significant at both time points, and the difference between OpaI+ and ΔopaBEGK statistically significant at 60 minutes (Figure 3.3D). While there was a trend towards more phagocytosis of OpaI+ than OpaF+ Gc as time progressed, this was not statistically significant.

The results with imaging flow cytometry were extended using an immunofluorescence assay that reports the percent of cell-associated bacteria that are intracellular (217). Here we focused on the nonvariable Opa strains so that the potential confounder of phase variation was removed. At 60 minutes post-infection, there were no significant differences among the Opa-expressing Gc strains in the percentage of neutrophil-associated bacteria that were phagocytosed (Opa50nv: 56%, Opa54nv: 52%, OpaDnv: 67%) (Figure3.3E).

However, the percentage of intracellular Opaless bacteria (44%) was significantly lower than OpaDnv, in keeping with prior reports (Figure 3.3E) (57, 218).

Taken together, these results indicate that Gc expressing different Opa proteins differentially interact with human neutrophils. However, once bound to neutrophils, Opa+ Gc are readily phagocytosed, regardless of which receptor(s) they engage.

3.2.3 Survival of Gc from Primary Human Neutrophils is Modulated by Bacterial Opa Expression Profile

We examined how the association and phagocytosis of different Opa-expressing Gc affected bacterial survival from neutrophils, by enumerating CFU of bacteria from neutrophil lysates as a function of time (116). First examining the nonvariable strains, similar CFU of Opaless, OpaDnv, Opa54nv, and Opa50nv were recovered after 30 minutes of neutrophil exposure (Figure 3.5A).

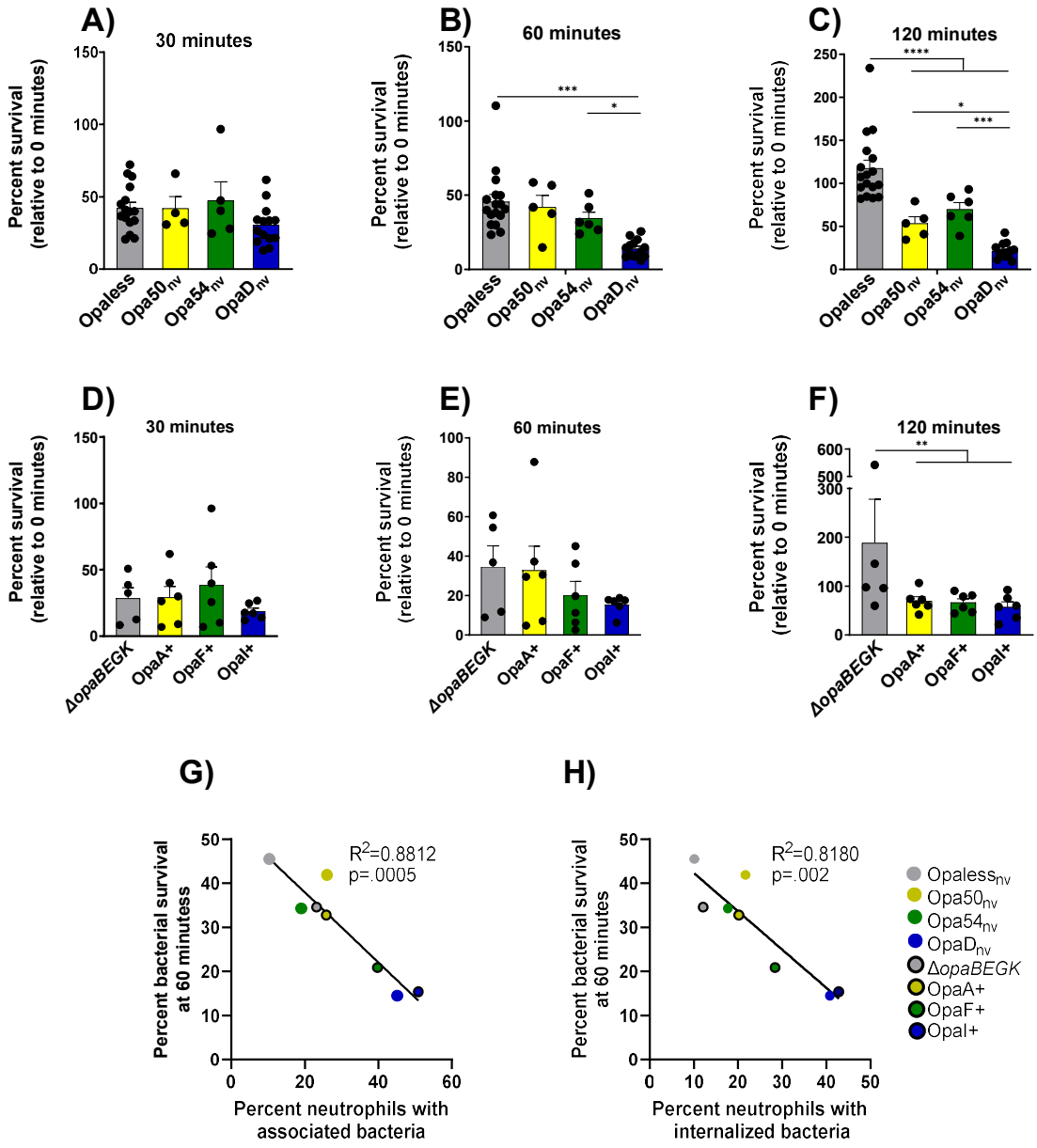


Figure 3.5: Differential survival of Gc of different Opa expression states after exposure to primary human neutrophils

Adherent, IL-8 treated neutrophils were synchronously exposed to constitutively expressed, nonvariable (A-C) or phase-variable (D-F) Gc of the indicated Opa profile. Colors match the receptor-binding profile of each strain as in Figure 10B. At 30 (A, D), 60

(B, E), and 120 (C, F) minutes post-infection, neutrophils were lysed, and CFU of Gc were enumerated from the lysates. Results are expressed as the average percent of CFU at that time point divided by the CFU at the start of the experiment (0 min) ± SE. For n≥4 biological replicates. Statistical comparisons were by two-way ANOVA with post-hoc Tukey multiple comparisons 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 R2 and P values are reported on the graphs.

However, after 60 minutes, fewer CFU of OpaDnv Gc were recovered compared with any of the other strains, with the differences from Opaless and Opa54nv Gc being statistically significant (Figure 3.5B). By 120 minutes post-infection, the recovery of OpaDnv Gc was significantly less than any of the other comparator strains (Figure 3.5C). We also saw outgrowth of Opaless at 120 minutes post-infection, 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 Gc followed the same trends as the Opa-nonvariable strains across all the time points tested, with no differences among *ΔopaBEGK*, OpaA+, OpaF+, and OpaI+ at 30 minutes (Figure 3.5D). While more CFU of *ΔopaBEGK* and OpaA+ Gc were recovered at 60 minutes compared with OpaF+ or OpaI+ bacteria, these differences were not statistically significant (Figure 12E). By 120 minutes, *ΔopaBEGK* was recovered in significantly greater numbers than any of the other Opa+ bacteria (Figure 3.5F). Interestingly, the CEACAM-binding profile did not directly correlate with bacterial susceptibility to neutrophils: while the CEACAM1 and CEACAM3 binding OpaDnv 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

to neutrophils and survival of the bacteria, as well as internalization into neutrophils and survival of bacteria (Figs. 3.5G and H).

3.2.4 Opa-receptor Interactions do not Affect the Survival or Maturity of Phagosomes Containing Opa+ Gc 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+ Gc survival from neutrophils. First, we found that Opa-expressing Gc, once phagocytosed, exhibited similarly low intracellular survival in neutrophils, as determined based on their permeability to propidium iodide (219). Extracellular Gc exhibited greater viability than intracellular bacteria, as previously reported (57), but there was no measurable difference in viability based on Opa expression in either the intracellular (Figure 3.6A) or the extracellular compartment (Figure 3.6B).

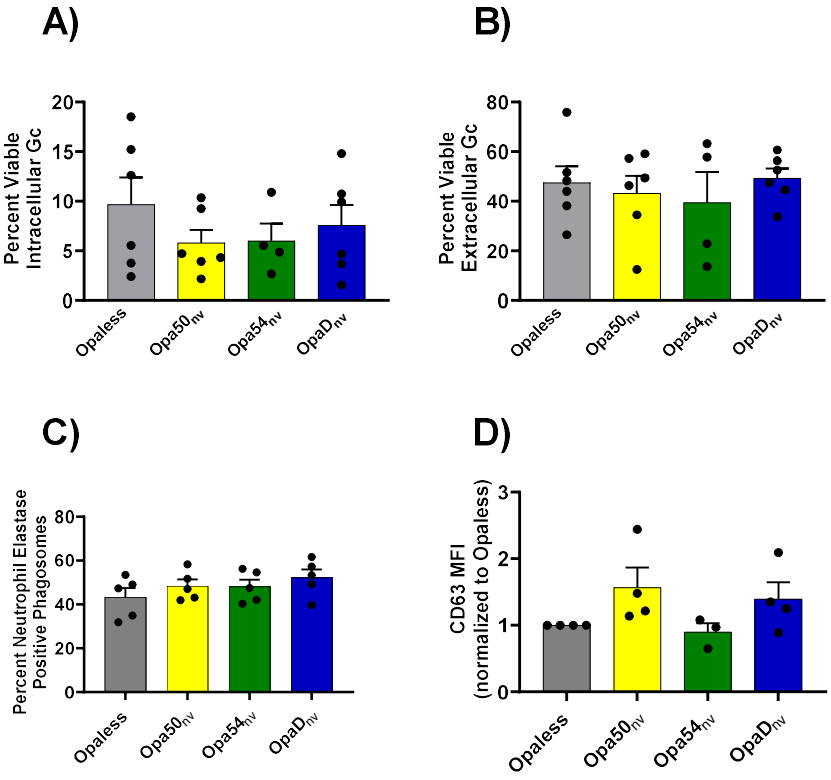


Figure 3.6: Opa expression state does not affect viability of Gc in the intracellular or extracellular compartments of neutrophils, or the release of neutrophil primary granules

A-B) Gc were incubated with adherent, IL-8 treated primary human neutrophils for one hour. Infected neutrophils were exposed to AF647-coupled soybean lectin to recognize extracellular Gc, then exposed to BacLight LIVE/DEAD viability dyes in the presence of saponin. The percent of viable (SYTO9+) Gc in the intracellular (A) (AF647-) and extracellular (B) (AF647+) compartments was quantified for n≥4 biological replicates. There were no statistical differences among strains in either compartment using one-way ANOVA with Tukey's multiple comparisons test.

C) Adherent, IL-8 treated neutrophils were exposed to CFSE-labeled Gc for 60 minutes. Cells were fixed and stained without permeabilization with rabbit anti-Gc antibody, followed by AF647-coupled goat anti-rabbit IgG, to label extracellular Gc. Cells were re-fixed and exposed to mouse anti-neutrophil elastase IgG followed by Alexa Fluor 555-coupled goat anti-mouse IgG. The percentage of intracellular (CFSE+ AF647-) Gc in neutrophil elastase-positive phagosomes was quantified. Results are from n>3 biological replicates. There were no statistically significant differences by one-way ANOVA with Tukey's multiple comparisons test.

D) Adherent, IL-8 treated neutrophils were exposed to the indicated strains of Gc for 60 minutes. 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≥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 comparisons test.

Next, we evaluated the maturity of the phagosome in which the phagocytosed Gc were found, based on acquisition of the primary granule protein neutrophil elastase (57). At 60 minutes, all Opa+ Gc, regardless of receptor-binding profile, resided in phagosomes of similar maturity (Figure 3.6C), with a trend towards reduced phagosome maturation for Opaless Gc, as previously reported ($p=0.08$) (57). There was also no significant difference in primary granule exocytosis in response to exposure to the different Opa+ Gc variants, as reported by mean CD63 fluorescence intensity normalized to Opaless using flow cytometry (Figure 3.6D).

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

3.2.5 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 of OpaI+ bacteria recovered from neutrophils 120 minutes, relative to 30 min and 60 minutes (Figure 3.5F), given that OpaI binds to CEACAMs 1 and 3 and is readily bound and phagocytosed by neutrophils (Figure 3.3C-D). To examine this Opa expressor further, we generated a strain of Gc with a constitutively expressed, nonvariable

opaI in the OpaD locus (OpaInv) (see materials and methods for details). While similar CFU of OpaInv and OpaI+ Gc were enumerated at earlier times of infection, at 120 minutes significantly fewer OpaInv were recovered compared to OpaI+ bacteria (Figure 3.7A).

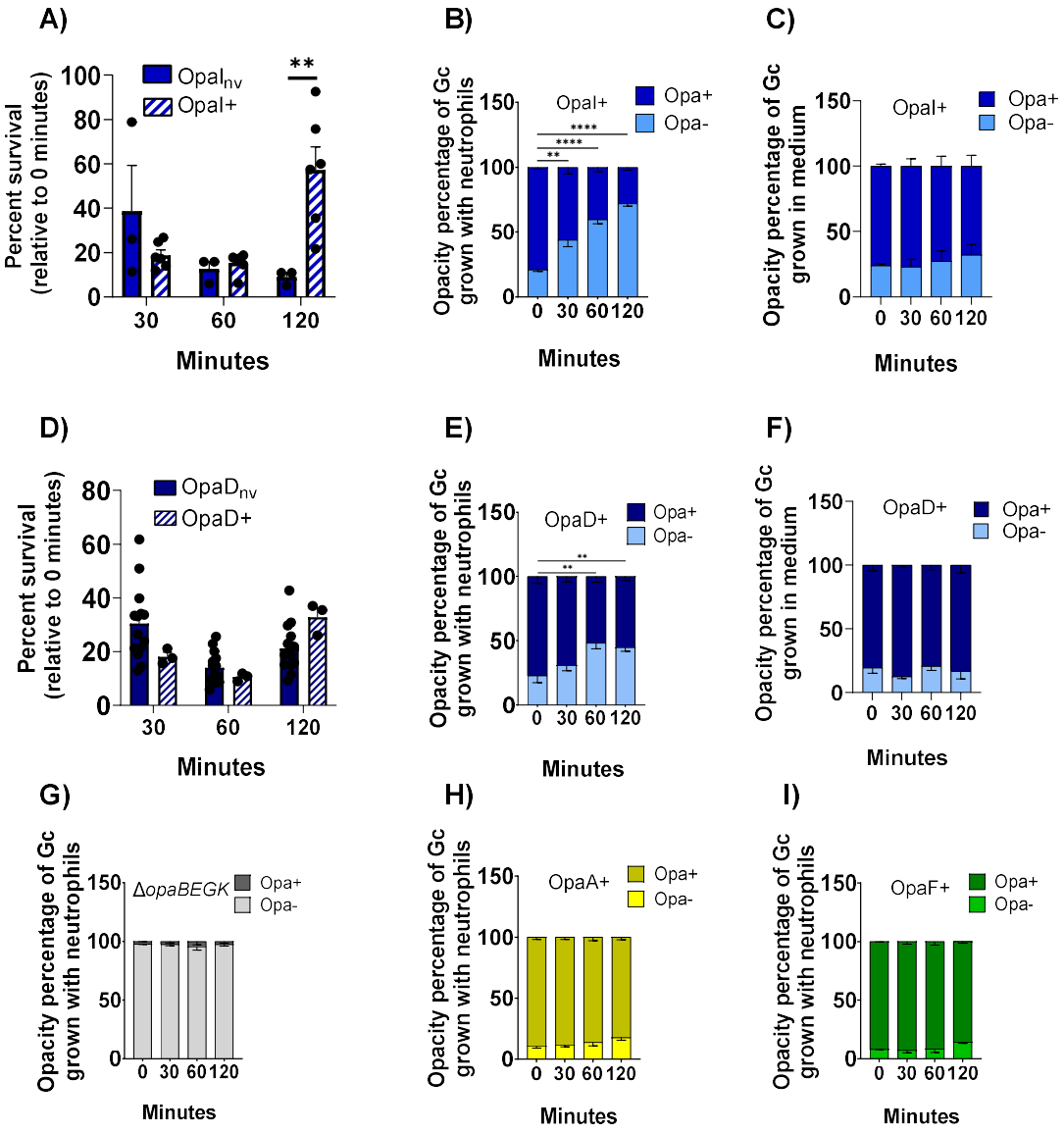


Figure 3.7: Neutrophil challenge selects for phase-OFF expression of Opa proteins that drive association of Gc with neutrophils, increasing overall survival of the population of Gc

Gc that were constitutively expressing (nv) or phase-ON (+) for OpaI (A) or OpaD (D) were exposed to adherent, IL-8 treated primary human neutrophils, and CFU were

enumerated from bacterial lysates over time as in Figure 3. Bacterial survival is expressed relative to the CFU enumerated at time 0.

At each time point from A and D, opacity phenotype of the enumerated CFU was visually inspected and recorded. Results are reported as the percent 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 OpaI+ (B) and OpaD+ (E) were observed

The same starting cultures of OpaI+ (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 OpaI+ (C) or OpaD+ (F)

ΔopaBEGK (G), OpaA+ (H), and OpaF+ (I) Gc were exposed to neutrophils, and the opacity phenotypes of the CFU recovered at each time point were plotted as in B,E. Statistical comparisons were by two-way ANOVA with post-hoc Tukey multiple comparisons test, with the following pairwise significances: *$p<0.05$, **$p<0.01$, ****$p<0.0001$.

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 media control (RPMI with 10% FBS). The percent of Opa+ colonies in the OpaI+ inoculum (time 0 minutes) with neutrophils was similar to the percent in the media control, as expected (Figure 3.7B, 3.7C). However, in the presence of neutrophils, the percent of Opa+ colonies in the population significantly decreased over time, such that by 120 minutes, 72% of the colonies associated with neutrophils were Opa- (Figure 3.7B). In the media control, 76% of the starting OpaI+ population was Opa+, and this percentage did not significantly change over time (Figure 3.7C). 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 minutes of neutrophil exposure (Figure 3.8).

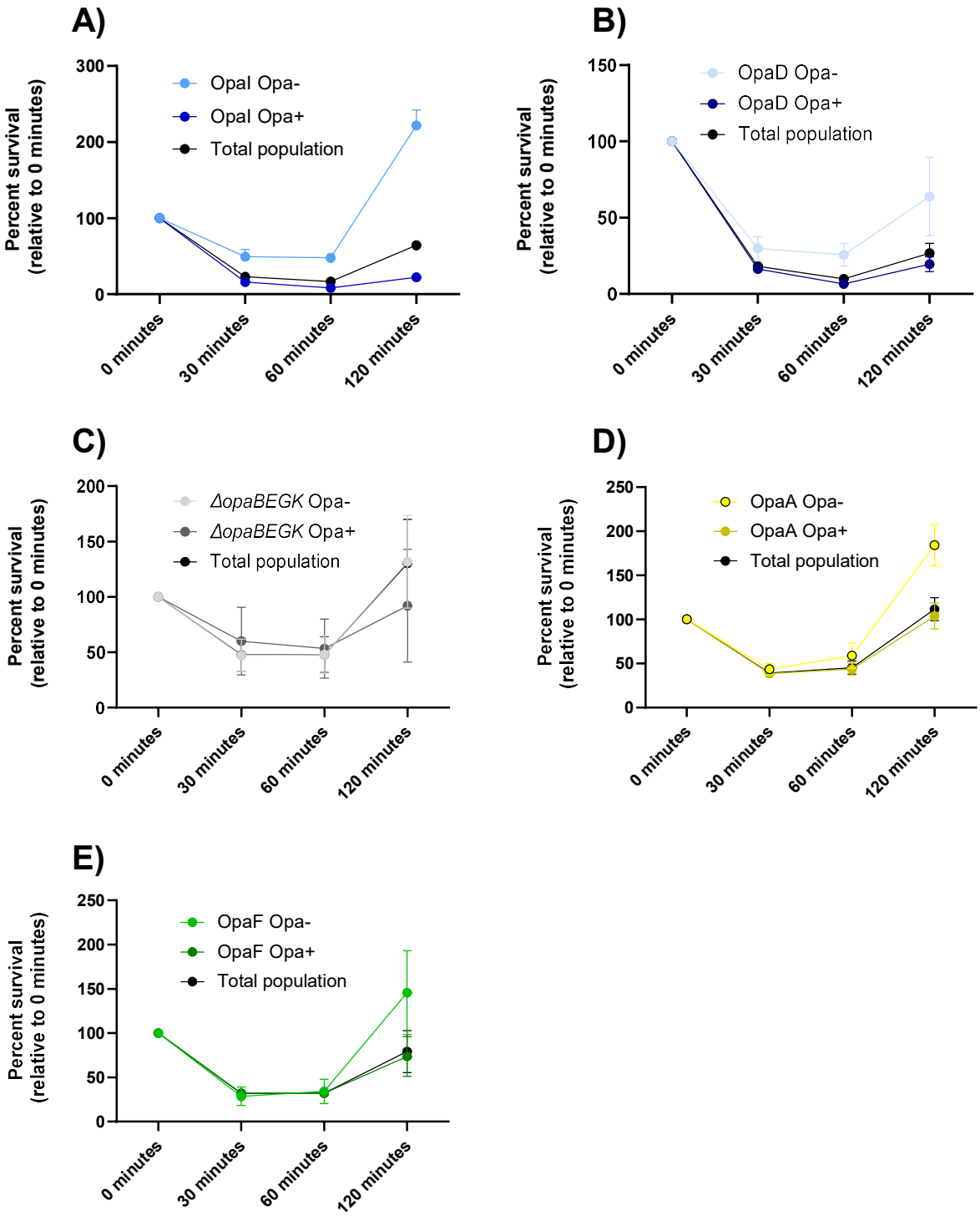

A)
Percent survival (relative to 0 minutes)
300
200
100
0
OpaI Opa-
OpaI Opa+
Total population
0 minutes
30 minutes
60 minutes
120 minutes
B)
Percent survival (relative to 0 minutes)
150
100
50
0
OpaD Opa-
OpaD Opa+
Total population
0 minutes
30 minutes
60 minutes
120 minutes
C)
Percent survival (relative to 0 minutes)
200
150
100
50
0
ΔopaBEGK Opa-
ΔopaBEGK Opa+
Total population
0 minutes
30 minutes
60 minutes
120 minutes
D)
Percent survival (relative to 0 minutes)
250
200
150
100
50
0
OpaA Opa-
OpaA Opa+
Total population
0 minutes
30 minutes
60 minutes
120 minutes
E)
Percent survival (relative to 0 minutes)
250
200
150
100
50
0
OpaF Opa-
OpaF Opa+
Total population
0 minutes
30 minutes
60 minutes
120 minutes

Figure 3.8: Survival of Opa phase ON and phase OFF Gc within a variable Opa-expressing population of Gc after neutrophil challenge

The results from Figure 3.7 for bacteria of the indicated starting Opa profile were plotted as the percent survival of the Opa+ and Opa- populations within each culture after exposure to primary human neutrophils. Percent survival is the CFU enumerated at the indicated time point, divided by the CFU enumerated at time = 0 min x 100%. The percent survival of all Gc in the population is also included on each graph.

We asked if the same observation would be made for OpaD-expressing bacteria, which also highly associated with neutrophils (Figure 3.3A-B) and survived less well from neutrophils than the other Opa-nonvariable bacteria (Figure 3.5A-C). To do so, we isolated OpaD phase-ON (OpaD+) bacteria from the *ΔopaBEGK* background and compared their survival to OpaDnv after exposure to neutrophils. OpaD+ trended towards surviving better than OpaDnv from neutrophils but was not statistically significantly different (Figure 3.7D). 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 minutes (77% Opa+ in the inoculum vs. 55% at 120 minutes) (Figure 3.7E). OpaD+ in the media control maintained its Opa+ status over time (Figure 3.7F). As with OpaI+, the Opa- bacteria outgrew the OpaD+ bacteria, skewing the survival of the OpaD+ population higher than what was seen for OpaDnv (Supp. Figure 3.8B).

Similar analyses were performed for the parent of the variable strains, *ΔopaBEGK,* and the OpaA+ and OpaF+ expressors. After exposure to neutrophils, > 95% of *ΔopaBEGK* colonies remained Opa- throughout the 120 minute infection period (Figure 3.7G). While there was a slight increase over time in the proportion of Opa- colonies in the OpaA+ (Figure 3.7H) and OpaF+ (Figure 3.7I) population 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 media control, with *ΔopaBEGK* remaining predominantly Opa-, and OpaA+ and OpaF+ mostly Opa+ (Figure 3.9A, B, C).

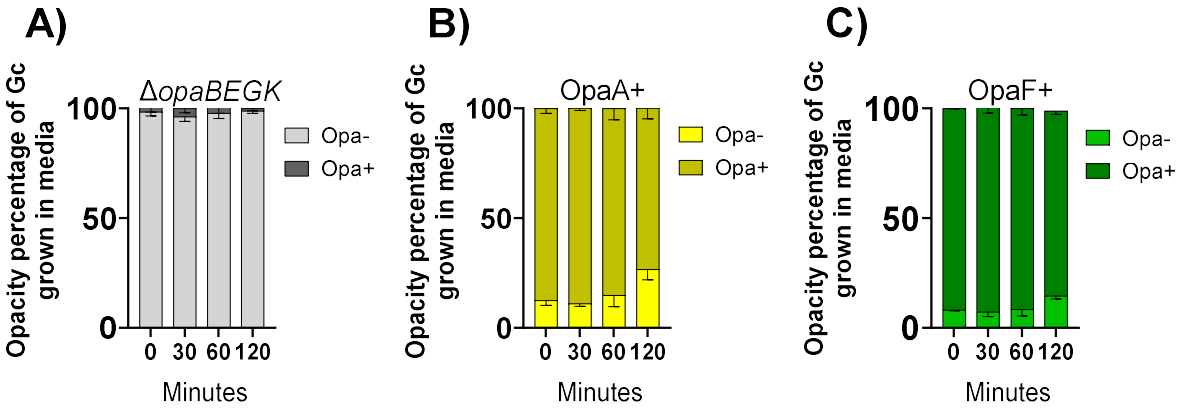


Figure 3.9: Opa phase-variable Gc grown in medium without neutrophils maintains the starting Opa phenotype

The same cultures of Gc of the indicated Opa variable expression states used in Figure 5 were inoculated into RPMI with 10% FBS. At the indicated time points, Gc CFU were enumerated based on opacity profile. There were no significant changes in Opa expression for ΔopaBEGK (A), OpaA+ (B), or OpaF+ (C) as determined by two-way ANOVA with post-hoc Tukey multiple comparisons test.

While there tended to be more Opa- than Opa+ colonies in these variable Opa populations 120 minutes post-neutrophil infection, this change did not have a major impact on the overall survival of the ΔopaBEGK, OpaA+, and OpaF+ populations (Figure 3.8C-E).

Taken together, these data indicate that the ability to phase-vary Opa expression is advantageous for Gc. A heterogeneous population allows the bacteria to avoid phagocytic killing by neutrophils, specifically in cases where the Opa+ bacteria are rapidly and efficiently phagocytosed.

3.3 DISCUSSION

Opa proteins comprise an important family of adhesins and invasins for Gc, and most Gc 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 compared with those lacking Opa expression. In this work, we investigated how differential interaction with primary human neutrophils affects Gc survival in an Opa-dependent manner. To do so, we used two panels of isogenic Gc, 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 Gc after exposure to adherent, IL-8 treated primary human neutrophils. Instead, survival

is impacted by the degree to which Opa expression affects Gc 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 Gc, regardless of which Opa protein(s) the bacteria in the population express.

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. Gc with nonvariable Opa expression, in an Opa-deleted background (Opaless), allowed for exact control of which Opa protein was expressed on Gc. Using predominantly expressing Opa+ Gc in a background with limited Opa variation capacity (ΔopaBEGK) 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 Gc 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 Gc in the infection mix over time. Thus, the ability of Gc 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 (173). Neutrophils constitutively express CEACAM3 at a relatively low level, regardless of their activation status, while CEACAM1 expression is upregulated with exposure to cytokines (220). The non-CEACAM binding strains could bind to 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 Gc expressing OpaD and OpaI strongly associate 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 OpaInv and OpaDnv, where the nonvariable opa allele is under 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 us better understand how the Gc 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 Gc, 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 (51, 221). In contrast, CEACAM3 contains an ITAM domain and has been shown to be activating (48, 55). Signaling downstream of CEACAMs involves recruitment of kinases leading to p47phox activation and consequent NADPH oxidase assembly (60, 61). 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 Gc expressing a CEACAM1-only binding Opa protein (222). Interaction of HSPGs, the secondary receptor of Opa proteins, with β-integrins has been shown to activate neutrophils in a similar manner (223). 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 Gc (222). Our work is in agreement in that we demonstrate here that primary granules mobilize and are delivered to both the Gc-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 (57). While we do not have a direct explanation for this difference, variation in human subjects' neutrophils or other as-yet 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 (59). 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 (175, 176, 188).

Some viruses are also able to utilize CEACAM1 as a cellular receptor (224). Similar to Gc, none of the known species of CEACAM-binding bacteria have an adhesin that binds solely to CEACAM3. Our data and others show that Gc with the ability to bind to CEACAM3 are more likely to be phagocytosed and killed by neutrophils (44, 45, 55–57, 189, 222, 225, 226). 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 Gc inoculum is predominantly expressing phase-variable CEACAM1 or CEACAM3 binding OpaI or OpaD proteins, which is not observed in bacterial populations expressing Gc 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 (165, 166, 227). In contrast, the enhanced phagocytosis of Opa+ Gc by neutrophils leads to decreased bacterial survival, as we and others have shown. In addition, the predominance of Opa-expressing Gc 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+ Gc are more sensitive (170). Along these lines, we reported that OpaD+ Gc is more sensitive than Opaless to killing by bactericidal/permeability-increasing protein (57). Hormonal changes may also affect how well Gc survives inside host cells (228).

Given the competing needs of Gc to colonize epithelial surfaces, yet avoid clearance by soluble and cellular immune effectors, phase variability of Opa proteins is advantageous to Gc on a population level. In particular, as uncovered in this study, the phagocytic and antimicrobial activities of neutrophils drive selection in the population for Gc that have phase-varied off expression of CEACAM3-binding Opa proteins, because of the enhanced phagocytic killing of the CEACAM3 expressors. However, Gc expressing Opa proteins that are less rapidly phagocytosed and killed do 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 Gc population to constantly test its environment in order to maximize their ability to colonize while avoiding immune clearance. These results provide one explanation for why human gonorrheal exudates commonly contain Opa+

Gc, 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 Gc in its obligate human hosts.

3.4 MATERIALS AND METHODS

3.4.1 Bacteria Used in this Study

All Gc 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 (105, 143). Opaless (*ΔopaA-K*) and OpaDnv (Opaless with a constitutively expressed, non-phase-variable *opaD* allele in the *opaD* locus) were described previously (105). OpaInv and Opa50nv were created in a similar manner to OpaDnv, with the non-phase-variable genes placed into the opaD locus. Opa50nv has been previously described (191). OpaInv was created by transforming into Opaless a synthesized *opaI* with the OpaDnv non-variable 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. Opa54nv was created by cloning a constitutively expressed, non-phase-variable version of opa54 from strain MS11 (gift of S. Gray-Owen,

University of Toronto) (229) into the pKH35 complementation plasmid (230), then incorporating the allele between lctP and aspC in Opaless by spot transformation and selection using chloramphenicol (0.5 μg/mL) (173). In Opa54nv, Opa54 expression is induced by growing the Gc in the presence of 1 mM IPTG. ΔopaBEGK, 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 (105). Predominantly OpaA+, OpaF+, and OpaI+ expressors in ΔopaBEGK 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) (231). Western blotting was similarly used to assess the Opa-predominance of the ΔopaBEGK population. The phase variable ¬opaA, opaF, and opaI sequences were extrapolated from the FA1090 genome sequence using the genomic locations previously reported (105). *opaD* (105), *opa50* (191), and *opa54* (173) sequences were published previously and confirmed after introduction into Opaless by DNA sequencing.

3.4.2 Bacterial Growth Conditions

Gc were grown overnight on gonococcal medium base (GCB; Difco) plus Kellogg's supplements (232) at 37° C with 5% CO2. Gc was grown in rich liquid

medium (GCBL) with Kellogg's supplements overnight with rotation at 30 °C, then back diluted twice and grown with rotation at 37 °C as previously described (217). Piliated Gc were enriched at the final dilution by collecting naturally sedimented bacteria for transfer into fresh medium. Opa54nv was grown in the presence of 1 mM IPTG in all liquid conditions.

3.4.3 CEACAM Binding of Opa+ Gc using Imaging Flow Cytometry

GST-tagged N-terminal domains of human CEACAM1 (N-CEACAM1) and CEACAM3 (N-CEACAM3) were purified as in (173). Opa+ Gc or the ΔopaBEGK parent bacteria (1x108 CFU/mL) were incubated with GST-tagged N-CEACAM (N-CEACAM1, N-CEACAM3) for 30 minutes at 37°C with end-over-end rotation. Gc incubated without any N-CEACAM was used as a negative control. Gc was then washed and stained to detect the presence of CEACAM with anti-GST antibody, as previously described (173). Bacteria were then resuspended in 2% paraformaldehyde with 5 μg/mL DAPI. Bacteria were processed using the ImageStreamX 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, the AF488 expression (173). The binding profiles of Opa50nv, Opa54nv, and OpaDnv were previously reported (173) as percent of the bacterial population positive for GST. For each sample, at least 40,000 cells were analyzed.

3.4.4 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 #13909). Neutrophils were isolated via dextran sedimentation followed by a Ficoll gradient as previously described (217). Neutrophils were resuspended in Dulbecco's PBS (without calcium and magnesium; Thermo Scientific) containing 0.1% dextrose and used within two hours of isolation.

3.4.5 Neutrophil ROS Production

Neutrophils (2x105) were resuspended in Morse's defined medium (MDM; (233)) in the presence of 20 μM luminol. Gc were added at a MOI of 100. Luminol-dependent chemiluminescence was measured every three minutes for one hour on a VICTOR3 Wallac luminometer (Perkin-Elmer) as previously described (60). One representative of ≥ 3 biological replicates is presented. Uninfected, untreated neutrophils were used as a negative control in each experiment (60).

3.4.6 Imaging Flow Cytometric Analysis of Bacterial Association with and Internalization by Neutrophils

Gc was labeled with Tag-it Violet™ Proliferation and Cell Tracking Dye (TIV) (Biolegend) in PBS with 5 mM MgSO4 for 15 minutes 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 and pretreated with IL-8. At the indicated time points, cells were fixed with 4% paraformaldehyde in PBS and removed from the coverslips by gentle scraping as previously described (216). 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 ImageStreamX Mk II imaging flow cytometer and analyzed with INSPIRE® and IDEAS® v. 6.2 Software packages (Luminex Corporation). Gating was completed as previously described (216). Briefly, focused, single cells were gated for low DL650, then spot counted for TIV+ Gc. Results are reported as the percentage of the neutrophil population with at least one bacterium that is associated (TIV+) (bound or internalized).

3.4.7 Gc Survival in the Presence of Primary Human Neutrophils

Neutrophils were treated with 10 nM human IL-8 (R&D) in Roswell Park Memorial Institute 1640 medium (RPMI) with 10% fetal bovine serum (FBS) at 37˚C with 5% CO2 and were allowed to adhere to 13 mm diameter plastic cover slips (Sarstedt) for at least 30 minutes prior to infection. Mid-logarithmic phase Gc were 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 (217). Results are reported as the CFU enumerated at the indicated time point, divided by the CFU associated with neutrophils at time 0 min x 100%.

3.4.8 Determination of Intracellular and Extracellular Bacterial Viability

Adherent, IL-8 treated neutrophils were exposed to Gc as for the bacterial survival assays, except Gc was added at MOI of 10. After 60 minutes, Gc were incubated for 10 min at room temperature in 0.1 M MOPS pH 7.2 + 1 mM MgCl2 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 non-viable Gc were detected using the BacLight LIVE/DEAD Viability Kit (Invitrogen) as previously described (219).

3.4.9 Neutrophil Phagosome Maturity

Adherent, IL-8 treated neutrophils were exposed to Gc at MOI of 1 as above, except bacteria were first labeled with carboxyfluorescein succinimidyl ester (CFSE) at 1:1000 in PBS with 5 mM MgSO4 for 25 minutes at 37 ˚C. After 60 minutes, cells were fixed and processed for immunofluorescence microscopy as in (58). Cells were blocked in PBS with 10% normal goat serum (NGS) for 10 minutes at room temperature. Extracellular Gc were 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).

3.4.10 Degranulation

Surface expression of primary granule markers was determined as previously described (122). Gc were incubated with primary neutrophils adhered to glass slides for one hour at 37 ˚C. Cells were then lifted for 10 minutes on ice using 5mM EDTA and washed twice in DPBS with 0.1% dextrose. They were then stained with PE-CD63 (Biolegend) for 30 minutes on ice, as an indicator of primary granule exocytosis. Isotype controls (Biolegend PE-IgG1) 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.

3.4.11 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 >50 individual bacteria/phagosomes were counted.

3.4.12 Statistics

For all experiments except for the chemiluminescence assay, results are depicted as the mean + standard error for > 3 independent experiments (different subjects' neutrophils, 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 ANOVA used for multiple comparisons for parametric data.

Chapter 4: DISCUSSION AND FUTURE DIRECTIONS

4.1 SUMMARY

In this dissertation, I sought to answer an outstanding question in the Gc field; why is there a selection for specific Opa proteins *in vivo,* and what drives that selection? Previous literature had shown that infection with Opa- Gc led to recovery of Opa+ Gc from infected individuals (156). Further studies have shown that specific Opas predominate in human challenge (171) and additionally, the overall population of Opa expressing Gc shows a selection for a specific CEACAM binding capability, in which a majority of collected isolates bind to CEACAM1, with a much smaller population binding both CEACAM1 and CEACAM3 (169). However, despite the understanding that there seemed to be advantage for Opa expression *in vivo,* what was leading to that selection was unknown.

Previous literature had shown that expression of Opa proteins, while useful for conferring a sustained infection, led to neutrophilic activation and bacterial death (45, 47, 57, 226). However, these studies did not consider which receptors the expressed Opa proteins were interacting with. Prior to this thesis, it was hypothesized that the signaling downstream of the transmembrane CEACAMs on neutrophils was the major factor in bacterial survival. The ITIM on

CEACAM1 suggested that binding CEACAM1 would lead to inhibitory signaling in neutrophils (234), allowing for survival, while Sarantis and Gray-Owen proposed that a crosstalk between CEACAM1 and CEACAM3 was occurring, activating the neutrophil in the same manner, regardless of what CEACAM was bound (222).

In this thesis, I was able to employ a system utilizing primary human neutrophils developed in the Criss lab. I built upon a bacterial genetic system generated by former lab manager Louise Ball in which all *opa* genes were deleted from strain FA1090**,** and into which individual non variable *opa* genes are added. I was able to determine the CEACAM binding capability of previously uncharacterized Opa proteins with fellow graduate student Lacie Werner and then determine the effect of that receptor binding by cloning and expressing genes of selected Opa proteins into the Opaless background. In addition, I also worked with predominantly phase-ON Opa+ bacteria in a minimally Opa variable background. I sought to understand how the differences in receptor binding affected neutrophil activation and bacterial survival. Overall, I found that Opa expression itself was not detrimental to Gc, but instead the main driver of bacterial killing was how well the bacteria associated with neutrophils.

My results show that Gc expressing Opa proteins that engage CEACAM1 and CEACAM3 showed greater association and phagocytosis by neutrophils than Opa+ Gc that do not bind CEACAM3 or Opa- Gc (Figure 4.1).

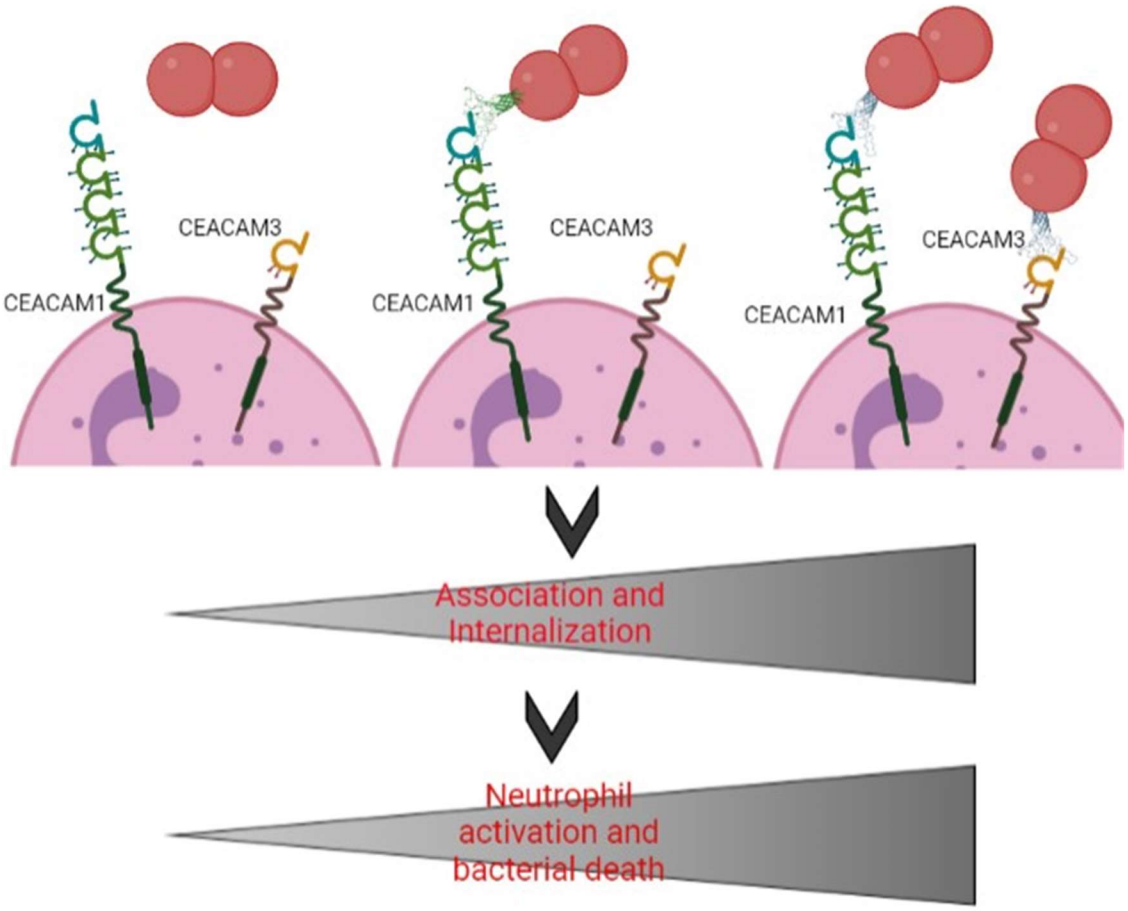


Figure 4.1 Increased association by CEACAM1 + 3 binding Opa+ Gc increases neutrophil activation and increased Gc population death

A model showing how Opa binding to CEACAM affects bacterial survival and neutrophil activation. Gc (pink diplococcus) interact with neutrophils via CEACAMs. Expression of Opa proteins leads to increased neutrophil association over Opa- Gc; the highest association occurs when Gc bind both CEACAM1 and CEACAM3. Increased association is correlated with increased neutrophil activation and bacterial death.

Interestingly, I found that once Opa+ Gc have associated with neutrophils, the bacterial outcome and activation of the neutrophil is similar independent of CEACAM binding; phagosome maturation, degranulation, and intracellular bacterial viability were similar independent of the Opa protein being expressed on the bacterial surface. Bacterial viability of Opa- bacteria was slightly increased over Opa+ Gc, implying that Opa- bacteria survive better as they are able to stay extracellular, as previously reported (57).

My data are able to advance the field of *Neisseria gonorrhoeae* by addressing how Opa+ Gc are able to colonize a host without being killed by the immune system. I show that neutrophil association is a major factor in bacterial killing, and while we were already knowledgeable about how Gc was able to evade neutrophil antimicrobial function, my findings show that Gc are able to reduce neutrophil association even before they need to evade immune killing.

The findings presented in this dissertation raise additional questions about the effects of Opa expression on interactions of Gc with neutrophils. First, how is Opa protein binding to neutrophils affected by biological conditions and binding affinity? Second, is neutrophil signaling different than the signaling that has been shown in other cell types, and are both CEACAMs 1 and 3 initiating the same signaling pathway? Third, are there differences in CEACAM expression at different activation states of the neutrophil, and do those differences play a role

in the Opa mediated interaction? Finally, are there other factors that affect the selection for Opa+ Gc in the human body?

4.2 BIOLOGICALLY RELEVANT NEUTROPHIL/GC INTERACTIONS

In this thesis, I investigated which CEACAMs each Opa protein was able to bind to and determined the outcome of neutrophil/Gc interactions dependent upon that binding capability. However, our binding data did not consider how likely those Opa/CEACAM interaction are in a cellular context. I also did not test how strong each of the interactions are, as well as how other receptors on the neutrophil surface affect the Gc's ability to bind with the intended CEACAM. In this section I will discuss considerations for future directions studying Opa/CEACAM interactions.

4.2.1 CEACAM Binding Capability of Opa Expressing Gc

In Chapter 2, we investigated the CEACAM binding ability of different Opa proteins to the known neutrophil and epithelial expressed CEACAMs. We used Gc non-variably expressing Opa proteins along with GST tagged recombinant N-CEACAM. Our study was able to specifically determine the CEACAM binding capability of the tested Opa proteins. Our assay was selective as well, as we did not see binding to non-Opa expressing bacteria or Gc expressing Opa50 which binds to HSPGs. While we are confident we are able to determine which CEACAM an Opa protein can bind to, it is important to note that there are

caveats to this assay, as CEACAMs *in vivo* that interact with Opas will be membrane bound to cells which may affect the interaction. There is some discrepancy in the Opa literature about which CEACAMs are bound by certain Opa proteins. For example, some literature shows that Opa59 of strain MS11 only binds CEACAM5 (189), while other literature shows Opa59 only binds CEACAM1 (185). Further, as shown in the thesis, Opa54 does not bind to CEACAM5, but other reports show that it can (189). These differences could be due to varied methods of testing the interactions. While we used a recombinant system and whole bacteria with locked Opa expression, other work has used cellular based systems with a single CEACAM expressed (166, 189).

4.2.1.1 Effects of Glycosylation on Opa/CEACAM Interactions

In human cells, CEACAMs are glycosylated and our reductive system did not utilize recombinant CEACAMs with any modifications. In humans, glycosylation occurs on the non-binding face of the CEACAM, so it is unlikely it would affect the Opa CEACAM interaction (185, 235). A recent study has shown that glycosylation does not affect the ability of CEACAM1 to dimerize (236), and as the CEACAM/CEACAM binding face is similar to that of the Opa/CEACAM interface, it is unlikely that Opa interactions would be affected by glycosylation. Given the knowledge that the structure of the Opa as well as interactions with other surface expressed components such as LOS affect the ability to bind

CEACAMs, testing how CEACAM modifications may affect Opa binding to CEACAMs be important in determining the biological relevance of our pulldown assay. A cellular model of CEACAM binding would be very useful in assessing these interactions.

4.2.1.2 Variation in Opa Proteins

In addition to different model systems being used, variable *opa* genes are under evolutionary pressure for diversification through mutation and recombination (237). Because of this, an Opa protein used in one study may differ from an Opa protein in another study though they are reported to be the same, as shown in differential CEACAM binding described in the section above. This highlights the importance of reporting the sequence of the tested *opas* being used in manuscripts, as even single nucleotide changes in the sequence could affect how each of the hypervariable loops is expressed, and how it interacts with other loops. Bos et al. reported that creation of an Opa protein with hypervariable loops from different genes with similar CEACAM binding profiles did not always lead to that same profile upon the creation of the new Opa (163). The affinity of binding could also be affected by nucleotide changes which leads to codon change. For these reasons, it is important that the sequence of the Opa being used is reported and compared to other Opa proteins. Creation of a library of Gc expressing an Opa with a known CEACAM binding profile with single

nucleotide mutations in the hypervariable regions could show the effects of recombination events, specifically on how the bacteria interact with CEACAMs. A library like this could also answer what sites in the *opa* sequence are important for CEACAM binding.

4.2.1.3 Binding Affinity to CEACAMs

Given the variability of CEACAM binding by Opa proteins and the knowledge that the binding is regulated by the structure of the protein more than the sequence, it is reasonable to question if there are different affinities for CEACAMs by Opa proteins. Previous work by Jen Martin of the Columbus lab in collaboration with our lab has shown via fluorescence polarization assays that both OpaD and Opa60 (strain MS11) have high nanomolar affinities for both CEACAM1 and CEACAM3 (191). These studies were done using non-tagged recombinant N-CEACAM testing for the percentage of the CEACAM that was bound to the surface of the Opa+ Gc, and while this is an interesting finding, it does not take into account how other neutrophil expressed components such as vitronectin, fibronectin, and heparan sulfate proteoglycans may affect the affinity of the Opa/CEACAM interaction, as can homo- and heterotypic CEACAM binding. Despite the caveat to this assay, it is still an important tool and could be used to determine the affinity of the non-CEACAM3 bind Opas used in this thesis. If the non-CEACAM3 binding Opa proteins showed lower affinity for

their receptors, this could serve as a potential explanation for the reduced association seen when using these Opa+ strains.

4.2.1.4 Opa Protein Expression on the Surface of Gc

In addition to the affinity to receptors, the amount of Opa expressed on the surface of the bacteria could also affect bacterial association with neutrophils. When working with the variable Opa+ strains, I was able to confirm which Opa was being expressed but were unable to test how many Opa proteins were expressed on the surface. Opa50 and OpaD were created in the same Opaless background, with the gene of interest added back into the *opaD* locus (105). This suggests that Opa50 and OpaD should have similar numbers of Opa expressed on their surface. Opa54 was created through complementation of a plasmid containing *opa54* into Opaless (173). The amount of Opa expressed on Opa54 may differ from the other non-variable strains.

To test the effects of the amount of Opa proteins expressed on the bacterial surface and its role in neutrophilic interaction, we could utilize the IPTG inducible nature of the pKH35 plasmid to alter Opa expression (205, 230). pKH35 was created as complementation plasmid to insert a portion of its sequence directly into the Gc genome. An inserted gene can be expressed through exposure to IPTG (205, 230). We can use this tool not only to test the effects of a protein for Gc, but also to potentially regulate how much of the protein is being

expressed on the bacterial surface by using differing concentrations of IPTG to induce different levels of expression.

Using a system of differential induction, we could quantify the amount of Opa expressed on the Gc surface using an α-Opa antibody on a Western blot and the Licor Odyssey, which would allow us to quantify the intensity of the Western blot band by fluorescence, indicating the amount of Opa being expressed. Once we confirmed that Opa expression differed dependent upon the IPTG concentration used, we could test how different levels of Opa expression affected neutrophil association and bacterial killing using the same assays performed in Chapter 3. I hypothesize that lower Opa protein expression would lead to less neutrophil association and decreased bacterial survival. This inducible system could also allow us to distinguish if some of the differences in reports about Opa receptor binding capability (discussed in section 4.2.1.2) is due to the amount of surface-exposed protein.

4.2.1.5 Opa Interactions with other Gc Surface Components

It is important to consider for all the Opas that interactions with other Gc surface expressed structures, specifically LOS, may play a role in which receptors they bind and what the affinity for that receptor is. We know that LOS affects how the Opa proteins look opaque grown on plates, so that interaction may also play a role in how the Opa proteins interact with CEACAMs and neutrophils. When

LOS is sialylated, it has been shown to prevent the Opa interaction with CEACAMs as well (161). Our model system used neutrophils isolated directly from human blood, but there is no human serum present in the infection, and no added sources for sialic acid for sialylation. Determining how sialylation status of the LOS on each of the Opa+ strains we generated affects that protein's interaction with CEACAMs and more so, its association to neutrophils would give a broader picture to how Opa/CEACAM interactions occur *in vivo*.

4.2.2 Binding to Neutrophils via non-CEACAM Receptors

Interestingly, it is still unknown how OpaA interacts with neutrophils. In this thesis, I show that OpaA does not interact with any of the Opa binding neutrophil expressed CEACAMs. It is possible that it interacts with HSPGs, similar to Opa50, or via a different receptor entirely. Overall, our work showed that OpaA+ showed similar association and survival phenotypes to Opa- Gc, indicating that OpaA+ Gc and Opa- bacteria could be interacting with neutrophils in the same manner.

4.3 NEUTROPHIL SIGNALING DOWNSTREAM OF CEACAMS 1 AND 3

While much previous literature has shown Opa-dependent signaling downstream of CEACAMs in either artificial conditions or other cell types (51, 182, 187), determining CEACAM signaling after interaction with Opa proteins in

primary human neutrophils would benefit the field by identifying key signaling pathways involved in the immune response to Gc.

4.3.1 CEACAM Signaling in Primary Human Neutrophils

Though I did not see differential activation of neutrophils downstream of CEACAM binding including phagosome maturation, degranulation, and phagocytosis, I have yet to investigate the signaling pathways that are stimulated downstream of each receptor in the context of primary human neutrophils infected with Gc. As described in Chapter 1, CEACAM1 recruits tyrosine phosphatase SHP1 while CEACAM3 recruits kinases leading to antimicrobial activity (52, 57) (Figure 1.1). Previous work investigating CEACAM signaling has taken place in cell types other than primary human neutrophils (51, 182, 187). Given our system and the availability of primary cells, testing CEACAM signaling would add to the field by showing the current *in vitro* works is recapitulated *ex vivo.* It would also give us insight into whether the pathways downstream of each CEACAM activate antimicrobial activity individually or if there is signal crosstalk, as has been previously reported (222). In lab, we can infect neutrophils with Gc expressing Opas with specific CEACAM binding capability, allowing us to understand how signaling downstream of each CEACAM occurs. Opa54 would give us signaling specifically of CEACAM1. While we do not have a CEACAM3 only binding Opa+ strain, we can elucidate

the signaling downstream of a CEACAM1 and 3 binding Opa expressing Gc based on current literature as well as comparisons to the Opa54 condition.

After infection, neutrophils can be fractionated so we can observe signaling at the membrane and in the cytosol specifically (60). As I measured similar antimicrobial activity and bacterial killing, we would hypothesize that overall, the signaling pathway would be similar regardless of if CEACAM1 alone, CEACAM1 and CEACAM3, or a non-CEACAM receptor are bound. Given the known pathways leading to phagocytosis and granule mobilization, I would expect to see phosphorylation or recruitment of Hck, Rac, PI3K, Syk, and others (238). Previous work has shown that there is a crosstalk between CEACAM1 and CEACAM3 (222), and given our data, that is a likely possibility.

My attempts at determining CEACAM signaling was thwarted by the difficulties of working with primary human cells, including high numbers of proteases that release upon cellular activation as well as the inability to genetically modify the cells to determine signaling downstream of a specific receptor. These reasons are why others have chosen to work with surrogate cells, but the results in neutrophils may not be recapitulated in those systems. Further, a transgenic mouse model with human CEACAMs expressed on murine neutrophils has been used (56), but this system did not include neutrophils expressing all the

CEACAMs found on human neutrophils, and does not take into account interactions with other human receptors on the surface of the cell.

4.3.2 Formation of the NADPH Oxidase

Despite similar outcomes for the bacteria and overall similar neutrophil activation, I did measure slight differences in neutrophil release of ROS in response to Opa+ Gc. I saw the most potent oxidative burst in response to OpaD, binding both CEACAM1 and CEACAM3. I observed the release of ROS when a CEACAM was bound by Gc, and less when the receptor was another binding partner. I also saw a delay in the release of ROS when using Opa54 which only binds CEACAM1. As discussed in Chapter 3, I saw similar levels of degranulation in response to all tested strains. This indicates that MPO, found in primary granules, is not affecting the outcome of our ROS assay, suggesting that the formation of the NADPH oxidase complex alone is what differs between Opa protein exposure. Understanding recruitment of and phosphorylation of signals downstream of either CEACEAM1 or CEACAM3 could help answer why I am seeing differences in ROS release.

Our initial hypothesis was that a more potent oxidative burst indicated that the neutrophils were activated in a more antimicrobial manner. Given our data indicating that association was the driving factor influencing bacterial survival, as well as observations of phagosome maturation and degranulation that were

similar regardless of CEACAM binding, we hypothesize that the differences were not due to a difference in overall neutrophil activation, but potentially the result of the amount of CEACAMs bound on the surface. My proposed experiment in section 4.2.1.4 could assist us in answering this question, as we could test the release of ROS from neutrophils after exposure to Gc expressing different amounts of Gc on its surface.

4.4 NEUTROPHIL RECEPTOR EXPRESSION

Another unanswered question that was raised because of this thesis: are there differences in CEACAM expression on neutrophils dependent upon activation state and do those differences play a role in the Opa-mediated interactions between neutrophils and Gc? A main hypothesis as to why I saw differential association to neutrophils dependent upon Opa expression is the availability of receptors on the surface of neutrophils.

4.4.1 CEACAM Expression on Neutrophils

CEACAM1 expression is upregulated on the surface of neutrophils after cellular activation, while expression of CEACAM3 is consistent throughout infection (220). While I found CEACAM1 and 3 binding Opa+ Gc to have higher neutrophil association at each timepoint tested, though by 60 minutes the non-CEACAM3 binding Opa expressing Gc strains showed association levels closer to that of the strains expressing CEACAM3 binding Opas, though there was still

a significant difference. This could be due to upregulation of CEACAM1, HSPGs, or other cellular receptors to which the bacteria could bind. Quantifying the amount of each CEACAM receptor on the surface of the neutrophils both before and after activation using Western blot or flow cytometry would allow us to better understand why there was differential association with Opa+ Gc. Our 60 minute time point testing neutrophilic association hints at an increase in receptor availability as well; I saw immediate high levels of association with the CEACAM1 and 3 binding Opa+ strains. Conversely, the non-CEACAM3 binding Opa+ strains saw an increase in association over the course of one hour.

Jerse et al showed that OpaF, a CEACAM1 only binding Opa, is commonly recovered early in infection in human challenge models. This would be advantageous to the bacteria as they would only be interacting with CEACAM1, and per my thesis, I saw a selective pressure from neutrophils only against those Gc expressing Opas that bound to both CEACAM1 and CEACAM3, whereas there was no selection against Gc expressing OpaF in the presence of neutrophils. Further, this temporal change in receptor expression could explain why there also seems to be a delay in both ROS when the Gc are expressing Opa54.

4.4.2 CEACAM1 Isoforms

The isoform of CEACAM1 expressed on the surface of neutrophils may also play a role in how well the neutrophils are able to kill bacteria. There are eleven

isoforms of CEACAM1, with the CEACAM1-4L isoform containing the ITIM found to be the most common on human immune cells (239). Dependent on which isoform is expressed in different individuals, the neutrophil activation and antimicrobial activity may differ as not all isoforms contain the ITIM.

Given our availability to a pool of human donors, we have the ability to define the CEACAM1 isoform expressed for an individual donor using Western blotting with an antibody specific for CEACAM1 and could define the isoform by the size of the band. With that knowledge, we could then compare the antimicrobial activity seen when using each isoform. Without activation of downstream signaling that leads to phagocytosis and granule mobilization, association alone may not be enough for bacterial killing. We utilize human donors for our studies, therefore have the capability to test a small population for their CEACAM1 isoform expression.

In addition to CEACAM1 isoforms, there have been reports of CEACAM3 isoforms (240, 241). One potential isoform has been suggested to be secreted isoform as it does not contain the ITAM cytosolic motif (240), while another has only been shown in a single patient with leukemia (241). As these isoforms are not well defined and seem to be connected to illness, it is unlikely they play a role in healthy individuals.

4.5 OTHER FACTORS AFFECTING SELECTION AGAINST OPA EXPRESSION

In addition to pressure of the presence of neutrophils upon Gc infection into a host, there are other factors within the context of a host that may affect the selection for specific Opa proteins *in vivo*. First, previous work from our lab has shown that OpaD+ Gc are significantly more susceptible to bactericidal/permeability-increasing protein (BPI) than Opaless Gc (57). BPI susceptibility testing of the other Opa proteins examined in this thesis could further reveal why expression of some Opas is disadvantageous to Gc. Secondly, the menstrual cycle has also been shown to affect Opa selection during infection (170). Dependent upon which phase of the cycle infection occurs, hormones alter the expression of proteases and immune effectors which affects viability of Opa+ Gc. Finally, the ability of Opa proteins to interact with CEACAMs expressed on epithelial cells may also affect how likely they are to sustain an infection in the host. Epithelial receptor binding may not contribute to bacterial killing, but if Gc is expressing Opa proteins that cannot interact with the expressed receptors, the bacteria will not stay in the host. Opa- bacteria have been shown to be less prevalent in clinical infection (156, 242). CEACAM1 is expressed in the upper genital tract (the endocervix and uterus) while CEACAM5 is expressed in the lower genital tract (ectocervix and vagina) (243). Dependent upon where an

infection happens in the genital tract, expression of different Opas may be advantageous to Gc.

4.6 OVERALL CONCLUSIONS

While neutrophils are normally successful as clearing infections from hosts, Gc is a master at evading these cells. Much previous work has shown that Gc is able to avoid the individual antimicrobial components of neutrophils such as NETs (99), ROS (110), and antimicrobial components such as cationic antimicrobial peptides (117), but my work shows that Gc is first able to reduce association with neutrophils dependent upon which Opa proteins it expresses, allowing for increased bacterial survival. In addition to avoiding the immune response of neutrophils, expressing Opa proteins that bind CEACAM1 will down regulate activation of T-cells (51), further dampening the adaptive immune response. These bacteria will also be able to stay in the host longer as they will be able to bind to endocervical cells (165, 166).

Given the overall conclusions I have drawn that high association with neutrophils leads to increased bacterial death, the question arises, why express Opa proteins at all? While my work does not address bacterial interactions with epithelial cells, other literature has shown that expression of Opa proteins that can bind the epithelial or endothelially expressed CEACAMs is imperative for a sustained infection (165, 166). In fact, interaction with CEACAMs via Opa

proteins on epithelial cells has been shown to reduce epithelial shedding (discussed in section 1.4.5) (165). Further, my work has shown that while expressing Opa proteins leads to more bacterial death than not expressing them, expressing Opa proteins that are not able to bind to CEACAM3 survive better at the population level than CEACAM-3 binding Opa+ Gc after exposure to neutrophils. Expression of Opa proteins seems to be a balancing act for Gc. The bacteria need to express the proteins so they can bind to and infect the host, but also need to make sure it is an Opa protein that will not lead to bacterial death via interaction with neutrophils. Further, Gc take advantage of the phase variability of these proteins such that they are able to have a very heterogenous population at any time during infection. Work from Ann Jerse's lab has shown that Opa expression changes over time but it is almost always variable within the population, with there very rarely being selection for only one Opa protein over the course of the infection (170, 171). This heterogeneity allows the bacterial population to be expressing multiple Opa proteins as one time which will allow the overall population to stay in the host and be able to transmit to a next person.

In addition to being phase variable, *opas* undergo continuous sequence variation through both recombination as well as mutation. This constant switching of the *opa* genes and subsequent Opa proteins provides Gc with a strong defense against the immune response of the host. There is limited capability of the

immune system to create protective immunity due to the constantly changing surface of Gc. Because Opa proteins are so variable, studying Gc and neutrophils is more than studying just the bacteria-cell interaction. Gc are successful as they act as a population that takes full advantage of its intrinsic ability to vary and survive a potent immune response.

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