Neisseria gonorrhoeae defense against human neutrophils and host-derived antimicrobials

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Abstract

Neisseria gonorrhoeae (Gc) is the sole causative agent of the disease gonorrhea. More than 100 million people are infected annually, and reported infection numbers are consistently on the rise. There are clinical isolates of Gc that have developed resistance to the latest third generation cephalosporins, which is the last recommended line of monotherapy for disease treatment, and there is still no available gonococcal vaccine.

Following transmission, Gc comes into contact with host mucosal surfaces. Host epithelial cells recognize the pathogen, and respond by releasing both antimicrobials to combat Gc and proinflammatory cytokines to alert the immune system. The cytokine gradient favors recruitment of large numbers of neutrophils to the site of infection. Neutrophils mount a robust immune response characterized by phagocytosis of Gc, production of reactive oxygen species by NADPH oxidase, formation of NETs and release of antimicrobials that are stored in cytoplasmic granules. Despite the potent immune response, Gc resists complete clearance, which suggests that Gc has defensive mechanisms against killing by neutrophils and host-derived antimicrobials at mucosal surfaces. Herein, I investigated the contribution of two important virulence factors, an LOS-modifying enzyme LptA and a clinically relevant antimicrobial efflux pump MtrCDE, for protection of Gc during interactions with human neutrophils. In addition, I explored the antigonococcal activity of a host-derived antimicrobial, β-defensin 22, and characterized its potential for therapeutic application.

Gc interactions with neutrophils are not well understood. Though several important observations by our lab and other groups have advanced our understanding, there are still many unknowns. I have identified that LptA and MtrCDE are important for protecting Gc from killing by neutrophils. Previously, LptA was only known to protect Gc from killing by the complement component of innate immunity as well as by the host-derived antimicrobial LL-37. Here, I show that LptA contributes

to Gc defense against several neutrophil-derived components including the α-defensin HNP-1, CAP37/azurocidin and the serine protease cathepsin G. I also demonstrate that LptA-expressing Gc manipulates neutrophil phagosome maturation by disrupting primary granule fusion with the phagosomal membrane. Interestingly, the neutrophil-derived antimicrobials that LptA defends Gc against are all stored in primary granules. I also demonstrate that MtrCDE protects Gc against neutrophil killing. Previously, MtrCDE was known to be involved in the export of a variety of structurally diverse toxic substrates, such as antibiotics, fatty acids, nonionic detergents, bile salts and host-derived LL-37, out of the bacterial cell. I observe that MtrCDE improves survival of Gc exposed to a degranulated supernatant containing a mixture of neutrophil-derived antimicrobials. In addition, I show that both MtrCDE and LptA protect Gc from the antimicrobial activity elicited by NETs. Lastly, I identify a β-defensin with antigonococcal activity, though Gc have been reported to be highly resistant to defensins. Defensins are abundant in the reproductive tract, the predominant environmental niche for Gc, and represent an alternative therapeutic option to combat Gc. Taken together, these results expand our understanding of the role of two important Gc virulence factors during interactions with human neutrophils. We are slowly uncovering the inner-workings of the extensive defensive arsenal that Gc has at its disposal to resist killing by neutrophils and epithelium-derived antimicrobials during infection.

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1. Chapter 1: Introduction

1.1 Gonorrhea: scope of infection

1.1.1 *Global burden of disease*

Gonorrhea is a growing threat to global health. The estimated number of annually infected individuals is consistently on the rise year-to-year. The most recent reports from the World Health Organization estimate over 106 million cases per year (World Health Organization, 2012); although, many cases go unreported and true disease numbers are significantly higher. The Gram-negative bacterium *Neisseria gonorrhoeae* (gonococcus or Gc) is an obligate human pathogen, and the sole etiologic agent of gonorrhea. In 2013, The CDC identified Gc as an urgent threat to public health due to an alarming rise in the incidence of multidrug-resistant clinical isolates that exhibit resistance to the latest third-generation extended-spectrum cephalosporins and last recommended line of treatment (CDC, 2013). Resistance to cephalosporins marked the advent of untreatable gonorrhea, and prompted the CDC to give Gc "superbug" status (CDC, 2011; Lahra *et al.*, 2014). There is also no available gonococcal vaccine, due to obstacles with development. Antibodies targeting Gc surface structures including porin, opacity-associated proteins and lipooligosaccharide have been shown to only elicit limited protection against re-infection (Gulati *et al.*, 2013). The primary obstacle has been the identification of a conserved surface structure that is neither serovar-specific nor phase-variable, and is an epitope that is expressed by the majority of Gc strains (Gulati *et al.*, 2013).

1.1.2 *Symptoms and complications of disease*

Gonorrhea is primarily transmitted through sexual contact, and presents as cervicitis in women and urethritis in men (Wiesner and Thompson, 1980). It is not uncommon for Gc to infect other mucosal surfaces including those in the pharynx, rectum and conjunctiva of the eye (Wiesner and Thompson,

1980). In about 1-3% of infected individuals, gonorrhea disseminates and causes arthritis-dermatitis syndrome, septicemia and bacteremia (Workowski and Levine, 2002). Female disease is frequently asymptomatic during the initial stages of Gc infection. Ascending Gc infection into the upper reproductive tract and fallopian tubes leads to tissue damage, scarring and chronic inflammation, and often results in secondary complications including pelvic inflammatory disease (PID), ectopic pregnancy and infertility (Wiesner and Thompson, 1980). Gonorrhea can also be vertically transmitted during childbirth, and is a leading cause of neonatal blindness in the developing world (Wiesner and Thompson, 1980). Male disease is primarily uncomplicated, and presents with symptomatic local inflammation and recruitment of neutrophils to the site of infection (Spence, 1983). Tissue damage caused by inflammation in the urethra can cause dysuria and purulent discharge (Edwards and Apicella, 2004). It remains unclear whether the morbidity and host damage incurred during Gc infection is driven by the host immune response, release of toxic components by Gc or a combination of the two.

1.1.3 *Gc interactions with the host mucosal epithelium*

During the early stages of infection, Gc must contend with host innate immune defenses protecting the mucosal epithelium (Figure 1). The epithelium is coated with a thick mucoid layer containing epithelium-derived mucin, secretory IgA, and antimicrobial peptides including defensins, cathelicidin, lysozyme and phospholipases (Quayle, 2002; Moal and Servin, 2006). Many of these components have antigonococcal activity; however, Gc expresses virulence factors to resist clearance solely by host epithelial defenses as I will describe below (*1.2.1 Epithelium-derived components*).

After traversing the mucosal layer, Gc uses type IV pili to initiate attachment to the epithelium (Merz and So, 2006). Opacity-associated proteins (Opa) on the Gc surface mediate intimate attachment with host carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) and heparin sulfate proteoglycans to drive further bacterial association, as well as internalization into epithelial cells

(Makino *et al.*, 1991; Kupsch *et al.*, 1993). Additional Gc adhesins have been implicated in host cell invasion, depending on the cell type. The Gc lipooligosaccharide (LOS) lacto-N-tetraose moiety can engage the host asialoglycoprotein receptor on urethral epithelia (Harvey *et al.*, 2008). Alternatively, Gc pilus and porin can act cooperatively to interact with complement receptor 3 (CR3) on the surface of cervical epithelia (Edwards *et al.*, 2002).

1.1.4 *Detection of Gc by the host innate immune system*

The innate immune system can detect pathogen-associated molecular patterns (PAMPs) on the Gc surface or that are shed by Gc into the environment through pathogen recognition receptors (PRRs) expressed by host cells. Host epithelial cells and sentinel immune cells such as macrophages, dendritic cells and T_H17 helper cells express an array of PRRs such as toll-like receptors (TLRs) and NOD-like receptors (NLRs) that can detect a range of diverse pathogen PAMPs (Makepeace *et al.*, 2001; Massari *et al.*, 2002; Fisette *et al.*, 2003; Van Vliet *et al.*, 2009; Feinen *et al.*, 2010). As an example, Gc LOS potently activates the TLR4/CD14/MD2 complex on the plasma membrane, while Gc porin and lipoprotein H.8 are recognized by endosomal TLR2 (Massari *et al.*, 2002; Pridmore *et al.*, 2003; Zughaier *et al.*, 2005; Van Vliet *et al.*, 2009; Feinen *et al.*, 2010). Gc also sheds peptidoglycan fragments during cell wall turnover, which can be detected by host cytoplasmic NLRs (Kaparakis *et al.*, 2010; Mavrogiorgos *et al.*, 2013). During infection with Gc, PAMP recognition by host PRRs drives activation of downstream signaling pathways in host cells that induces expression and subsequent release of several pro-inflammatory cytokines including IL-6, IL-8, IL-1β and TNF-α (Waage *et al.*, 1989; Fichorova *et al.*, 2001).

1.1.5 *Neutrophil recruitment*

The cytokine gradient produced by host cells strongly favors recruitment of polymorphonuclear leukocytes (PMNs or neutrophils) to the site of Gc infection (Figure 1). Urethral discharge from infected males and cervical exudate from infected females contain abundant neutrophils (Figure 2) (Evans, 1977;

Edwards and Apicella, 2004). Gc in these secretions is attached to and within neutrophils (Figure 2) (Ovcinnikov and Delektorskij, 1971; Farzadegan and Roth, 1975; Evans, 1977; Apicella *et al.*, 1996). Neutrophils are potently phagocytic innate immune cells and first responders to many bacterial and fungal infections (Borregaard, 2010). PMNs are capable of phagocytosing and killing Gc, yet viable bacteria can be cultured from the purulent exudate of infected individuals (Wiesner and Thompson, 1980). Despite a robust immune response, neutrophils fail to completely clear the infection. Resistant Gc can establish long-term colonization, which increases the potential for dissemination and transmission of disease.

1.1.6 *Phagocytosis and intracellular killing mechanisms*

Phagocytosis of Gc occurs through both opsonic and non-opsonic mechanisms (Discussed in more detail in section *1.4 Neutrophil phagocytosis of Gc*) (Groves *et al.*, 2008). Gc is internalized into neutrophil phagosomes that fuse with cytoplasmic granules (Figure 3). Granules are divided into four types based on their unique complement of protein markers. Primary (azurophilic) granules contain serine proteases, azurocidin, BPI, α-defensins and myeloperoxidase (MPO) (Pham, 2006). Secondary (specific) granules contain LL-37 (cathelicidin), lysozyme, components of the NADPH oxidase and CR3 (Pham, 2006). Tertiary (gelatinase) granules contain primarily gelatinase, but also contain lysozyme and NADPH oxidase components. Lastly, secretory granules contain largely PRRs, but preferentially fuse with the plasma membrane rather than the phagosome (Pham, 2006). NADPH oxidase on the phagolysosomal membrane produces reactive oxygen species (ROS) that are capable of killing microorganisms, although neutrophil oxidative killing mechanisms are not important *in vitro* for combating Gc (Criss *et al.*, 2009). However, several non-oxidative neutrophil granule components including serine proteases, cationic antimicrobial proteins (CAPs) and cationic antimicrobial peptides

(CAMPs) are important for killing Gc, and are discussed in more detail below (*1.2 Host-derived antimicrobials*).

1.1.7 *Extracellular killing mechanisms*

PMNs also use extracellular killing mechanisms to target microbes that are not phagocytosed. DNA-based neutrophil extracellular traps (NETs) immobilize Gc that are in proximity to PMNs. Localized granule fusion at the plasma membrane causes release of positively charged CAPs and CAMPs to decorate NETs. In addition, extracellular ROS are produced by NADPH oxidase at the plasma membrane. However despite these killing mechanisms, PMNs are largely ineffective at killing extracellular Gc *in vitro*, due to the contribution of important Gc virulence factors.

1.1.8 *Gc defenses against killing by neutrophils*

Gc possesses several important virulence factors that aid in defense against epithelium- and PMN-derived antimicrobials. Importantly, some of these factors contribute to intracellular and extracellular survival of Gc during interactions with PMNs. Below, I discuss the role of epithelium-derived antimicrobials for host defense from invading pathogens, and present results for a mucosal β-defensin having antigonococcal activity. I also describe the role of two important virulence factors for Gc defense against killing by neutrophils and their antimicrobial components.

1.2 Host-derived antimicrobials

In the following section, I detail important innate immune antimicrobial components that are produced by epithelial cells and neutrophils to protect the host from pathogenic microorganisms. Some of these antimicrobials are produced in both epithelial cells and neutrophils, and will be discussed in context for both cell types.

1.2.1 *Epithelium-derived components*

The epithelium is the first line of defense from invading pathogens. Epithelial cells form intracellular tight junctions to restrict transepithelial migration of foreign particles (Quayle, 2002). Specialized epithelial cells secrete mucus, which forms a dense, slippery layer composed primarily of water (90-98%) and mucin glycoproteins (Quayle, 2002). The mucus layer physically protects the mucosal epithelium, excludes pathogens, and provides a medium for secretory immunoglobulin, enzymes, and antimicrobial components released by epithelial cells (Quayle, 2002). Many types of antimicrobials including α- and β-defensins, cathelicidin, lysozyme and lactoferrin have been isolated from mucosal secretions (Quayle, 2002).

1.2.1.1 *Defensins*

Defensins are a family of small (2-10kDa) CAMPs with antimicrobial activity against Gram-negative and Gram-positive bacteria, fungi, protozoa and enveloped viruses (Quayle, 2002). Defensins are active from about 1-100μg/ml (0.5-50μM), and vary in their potency. Defensins have a conserved β-sheet structural fold stabilized by three intramolecular cysteine disulfide bridges (Quayle, 2002). There are three subfamilies of defensins: α-, β- and θ-defensins. α- and β-defensins differ primarily by spacing of their cysteine residues and by the pattern of their disulfide linkages (Liu *et al.*, 1997). θ-defensins are smaller cyclical peptides that are only known to be produced in monkey species (Pazgier *et al.*, 2006). Defensins are expressed as precursor peptides that need to be processed by host peptidases and proteases into their mature, biologically active forms (Moal and Servin, 2006).

The α-defensins are 29-35 amino acid residues in length, and contain three disulfide bridge linkages (Cys1-Cys6, Cys2-Cys4, Cys3-Cys5) (Moal and Servin, 2006). To date, six α-defensins have been identified. The first four α-defensins were isolated from human neutrophils, and were named human neutrophil peptides (HNPs) 1-4 (Quayle, 2002). Human α-defensins 5 (HD-5) and 6 (HD-6) were initially

found to be expressed by Paneth cells of the small intestine, and more recently HD-5 was also shown to be expressed in the endocervix and endometrium of the female reproductive tract (Quayle *et al.*, 1998). There is some evidence that α-defensins may also be expressed in the male reproductive tract, although these reports are less definitive (Patil *et al.*, 2005).

β-defensins are longer CAMPs that range from 38-90 amino acid residues in length, and have a disulfide bridge arrangement that differs from α-defensins (Cys1-Cys5, Cys2-Cys4, Cys3-Cys6). β-defensins are uniquely expressed by non-granular mucosal epithelial cells lining the respiratory, gastrointestinal and genitourinary tracts (Patil *et al.*, 2005). Genome-wide homology analyses have identified 39 β-defensin genes encoded in the human genome (Patil *et al.*, 2005). Most β-defensins are preferentially expressed in the male reproductive tract, with the exception of hBD-2 which is not expressed at detectable levels at this site (Patil *et al.*, 2005; Pazgier *et al.*, 2006). However, the physiological importance for the wide variety and region-specific expression of β -defensins in the male reproductive is not fully understood. β-defensins are also expressed in the female reproductive tract, although at significantly lower levels (Patil *et al.*, 2005). In addition to their antimicrobial activity, β-defensins structurally and functionally resemble chemokines, and many are involved in physiological processes including sperm maturation and capacitation (Patil *et al.*, 2005). Though, functions for most defensins beyond their *in vitro* antimicrobial activity have not been established.

1.2.1.2 *Cathelicidin*

Humans produce only one cathelicidin, LL-37/hCAP18, which is encoded by the *CAMP* gene (GenBankID 820). LL-37/hCAP18 was first identified in the bone marrow, and is expressed as an 18kDa precursor peptide (hCAP18) (Ramos *et al.*, 2011). The hCAP18 pro-peptide undergoes proteolytic processing by proteinase 3 to form the mature 37 amino acid residue peptide (Sørensen *et al.*, 2001). LL-37 is an α-helical CAMP, has no cysteine residues to form intramolecular disulfide bridges and has a

molecular weight of 4.5kDa. It is found in varying concentrations in cells, tissues and fluids throughout the body, but is predominately produced by neutrophils and epithelial cells (De Yang *et al.*, 2000). Cathelicidin has also been identified in breast milk, sweat, wound fluids, saliva, gingiva, testis, spermatozoa, seminal plasma and amniotic fluids (Ramos *et al.*, 2011). The expression of LL-37 is upregulated by several stimuli including proinflammatory cytokines, growth factors, nutrients and bacterial PAMPs (Ramos *et al.*, 2011).

LL-37 has broad antimicrobial activity against Gram-positive and Gram-negative bacteria and viruses, but has low activity against fungi (Ramos *et al.*, 2011). It has also been shown to limit the formation of bacterial biofilms (Overhage *et al.*, 2008). In addition to its antimicrobial activity, the CAMP contributes to host defense by neutralizing toxic LPS, acting as a chemo-attractant for innate and adaptive immune cells, and participating in wound repair. It disrupts interactions of LPS with CD14 and LPS binding protein (LBP) by binding to LPS with high affinity, which inhibits LPS-induced proinflammatory cytokine release by host cells (Larrick *et al.*, 1995; Brown *et al.*, 2011; Ramos *et al.*, 2011). Furthermore, it is chemotactic for mast cells, monocytes, T lymphocytes and neutrophils, which is mediated by engagement of LL-37 with the formyl peptide receptor-like 1 (FPRL-1) expressed on the surface of these cells (Ramos *et al.*, 2011). LL-37 also plays a role in wound repair by binding to FPRL-1 on endothelial cells to induce angiogenesis (Ramos *et al.*, 2011).

1.2.1.3 *Lysozyme*

Lysozyme is a small 14.5kDa protein recognized primarily for its muramidase activity. The enzyme hydrolyzes β-1,4 glycosidic bonds between N-acetylmuramic acid and N-acetyl-D-glucosamine residues of bacterial cell wall peptidoglycan. Lysozyme is produced in a wide variety of cells and tissues, and is present in nearly every bodily fluid. The highest concentrations of lysozyme are found in tears, gastric juice and breast milk (Wiesner and Vilcinskas, 2010). Lysozyme was named by Alexander Fleming

in 1922, when he observed the bacteriolytic activity of the enzyme in human secretions. In addition to its antibacterial activity, lysozyme also has broad activity against fungi, viruses and tumor cells by causing structural damage that often results in lysis of the target (Wiesner and Vilcinskas, 2010).

Although the bacterial target of lysozyme is known, the mechanism connecting lysozyme to bacterial death is not well-defined. The cell wall of Gram-positive bacteria is exposed to the external environment, and provides an accessible target for lysozyme hydrolysis. However, the peptidoglycan sacculus in Gram-negative bacteria is protected by an outer membrane that prevents penetration of the enzyme into the periplasmic space. Interestingly, lysozyme is a cationic protein (p*I* 9.3) that has been shown to kill bacteria independently of its enzymatic activity. Bactericidal activity against the Gram-positive *Strepococcus sanguis* is not diminished after catalytic inactivation of the enzyme by reduction of the intramolecular disulfide bonds (Laible and Germaine, 1985). Conversely, many bacterial species including *Staphylococcus aureus* are completely resistant to killing by lysozyme. These bacterial species are able to modify their cell wall peptidoglycan with additional *O*-acetyl groups, which increase bacterial resistance against lysozyme-specific muramidase activity (Bera *et al.*, 2006).

1.2.1.4 *Lactoferrin*

Lactoferrin is a mammalian iron-binding glycoprotein belonging to the transferrin family, and has a molecular weight of 80kDa. Transferrin is primarily localized to the bloodstream, whereas lactoferrin is found in neutrophils, as well as in exocrine secretions including milk, tears, nasal secretions, saliva, urine, uterine secretions and amniotic fluids (Sinha *et al.*, 2013). Lactoferrin has been shown to have antimicrobial activity against bacteria, viruses and fungi (Sinha *et al.*, 2013). It is a multifunctional antimicrobial protein that contributes to host defense in several ways. The iron-binding capability of lactoferrin is its best characterized function, and iron-sequestration by lactoferrin deprives pathogenic microorganisms of an essential nutrient required for their growth. Mammalian lactoferrins

are also significantly cationic. Several regions of the protein, including the positively-charged N-terminal helix, structurally resemble CAMPs. Many of these fragments have bactericidal activity, and some, such as the 25 amino acid residue peptide lactoferricin are naturally released by host proteolytic activity (Wiesner and Vilcinskas, 2010; Sinha *et al.*, 2013).

1.2.2 *Neutrophil-derived antimicrobials*

Epithelial cells detect invading pathogens by recognition of PAMP molecules shed by microorganisms. The PAMPs engage PRRs on the epithelial surface, and cause activation of signaling pathways that lead to increased expression and secretion of antimicrobials to control the infection. PAMP-recognition also induces increased release of inflammatory cytokines by epithelial cells. The resulting cytokine gradient is favorable for the recruitment of neutrophils to the site of infection. Neutrophils also become activated by detecting PAMPs. Activated neutrophils can engulf microorganisms into phagosomes. Neutrophils then mobilize cytoplasmic granules, which contain high concentrations of antimicrobial components, to fuse with internalized pathogens contained in phagosomes or to fuse with the plasma membrane to target extracellular pathogens (Figure 3). Activated neutrophils also form extracellular traps (NETs) that are decorated with antimicrobial granule contents (Discussed in *1.5 Neutrophil extracellular traps*).

1.2.2.1 *CAP37/Azurocidin*

The 37kDa cationic antimicrobial protein (CAP37) was first isolated from neutrophil extracts by Shafer *et al.* in 1984 (Shafer *et al.*, 1984). Later, Gabay *et al.* identified the same protein in neutrophil azurophil granules, and named it azurocidin (Gabay *et al.*, 1989). The protein is a member of the serine protease superfamily, but has mutations in two essential amino acids that make it catalytically inactive (Soehnlein and Lindbom, 2009). However, CAP37/azurocidin has protease-independent antimicrobial activity against Gram-negative and Gram -positive bacteria, and against fungal pathogens, which is due

to a cluster of eight arginine residues concentrated on loop3 and loop4 of the protein (McCabe *et al.*, 2002; Wiesner and Vilcinskas, 2010). CAP37/azurocidin is also unique among neutrophil cationic proteins, because it is present at high concentrations in both azurophil and secretory granules (Tapper *et al.*, 2002). Unlike HNPs, which are primarily delivered to the phagosome, about 90% of CAP37/azurocidin is released extracellularly during degranulation (Tapper *et al.*, 2002).

As with other CAPs and CAMPs, CAP37/azurocidin is a multifunctional protein that is also involved in host immune modulation. Neutrophils release CAP37/azurocidin from secretory granules early in degranulation, after which the CAP adheres to endothelial cells in the bloodstream (Soehnlein and Lindbom, 2009). Monocytes recognize deposition of CAP37/azurocidin, adhere to the endothelial cell layer and transmigrate across the cells towards the site of inflammation (Soehnlein and Lindbom, 2009). In addition, recruited monocytes and resident macrophages can be activated by CAP37/azurocidin, which augments release of proinflammatory cytokines and enhances surface expression of classical activation markers (Rasmussen *et al.*, 1996; Soehnlein *et al.*, 2008).

1.2.2.2 *CAP57/BPI*

The bactericidal/permeability-increasing protein (BPI) is a 55kDa CAP that is stored in azurophil granules of neutrophils (Elsbach, 1998). CAP57 was first isolated in neutrophil extracts by Shafer *et al*. in 1984 (Shafer *et al.*, 1984). The group later identified CAP57 and BPI as the same protein (Pereira *et al.*, 1990). The CAP belongs to the lipid-transfer protein family, and is a close relative to the LPS-binding protein (LBP). CAP57/BPI binds to the lipid A moiety of LPS with high affinity, and has potent antimicrobial and LPS-neutralizing activity against a variety of Gram-negative bacteria (Ooi *et al.*, 1991; Gazzano-Santoro *et al.*, 1995). Binding of BPI to the bacterial surface increases outer membrane permeability and disrupts energy-dependent cellular processes leading to eventual cell death (Mannion *et al.*, 1990).

1.2.2.3 *Cathepsin G*

The neutrophil serine proteases (NSPs) cathepsin G, neutrophil elastase, proteinase 3 and the more recently identified neutrophil serine protease 4 (NSP4) are prepackaged in azurophil granules of neutrophils (Perera *et al.*, 2012). Once released, the precursor NSPs undergo proteolytic processing by the dipeptidyl peptidase I into their mature form, and are optimally active in a neutral environment (Pham, 2006). Cathepsin G preferentially hydrolyzes peptide bonds after aromatic amino acid residues, while neutrophil elastase and proteinase 3 hydrolyze peptide bonds after a valine residue (Rock and Rest, 1988). NSPs are critical to host defense during infection. They hydrolyze surface proteins on invading microorganisms, and degrade extracellular matrix components to facilitate neutrophil migration through tissue. They also modulate the immune response through proteolytic processing of host chemokines, cytokines and cell surface receptors (Wiesner and Vilcinskas, 2010; Perera *et al.*, 2012).

Cathepsin G differs from other NSPs, because it is both highly cationic (p*I* 11.4) and has antimicrobial activity that is independent of its hydrolytic activity (W.M. Shafer, Onunka, *et al.*, 1986). Shafer *et al.* demonstrated that treatment with an irreversible serine protease inhibitor diisopropylfluorophosphate has no effect on the bactericidal activity of the serine protease against *N. gonorrhoeae* (W.M. Shafer, Onunka, *et al.*, 1986). The group also showed that synthetic peptides derived from cathepsin G (residues 1-5 and 77-83) retain antigonococcal activity (Bangalore *et al.*, 1990).

1.2.2.4 *HNPs*

The human neutrophil peptides (HNPs) 1-4 were the first α-defensins to be identified, are stored in azurophil granules and are specific to neutrophils (Quayle, 2002). Please refer to section *1.2.1.1 Defensins* for more a detailed description of α-defensins and their biological role in host defense.

1.2.2.5 *Cathelicidin*

Cathelicidin is stored as the precursor peptide hCAP18 in specific granules in neutrophils, and is proteolytically processed to the mature, biologically active LL-37 by proteinase 3 which is stored separately in azurophil granules (Sørensen *et al.*, 2001). Cathelicidin is discussed in greater detail in section *1.2.1.2 Cathelicidin*.

1.2.2.6 *Lysozyme*

Lysozyme is stored in large amounts in three neutrophil granule types: azurophil, specific and gelatinase (Wiesner and Vilcinskas, 2010). Lysozyme is discussed in greater detail in section *1.2.1.3 Lysozyme*.

1.2.2.7 *Lactoferrin*

Lactoferrin is primarily found in exocrine secretions, but is also stored in specific granules in neutrophils (Wiesner and Vilcinskas, 2010). Lactoferrin is discussed in greater detail in section *1.2.1.4 Lactoferrin*.

1.2.2.8 *Reactive oxygen species*

Neutrophils produce two distinct categories of antimicrobial components: oxidative and non-oxidative. Reactive oxygen species (ROS) are the only source of oxidative components in neutrophils. The neutrophil oxidative burst requires production of ROS by NADPH oxidase. In resting neutrophils, the NADPH oxidase $gp91^{phox}$/$p22^{phox}$ subunits are stored in specific and gelatinase granules, and $p40^{phox}$ $p47^{phox}$, $p67^{phox}$ subunits as well as the GTPase Rac2 are present in the cytoplasm. Activated neutrophils assemble the holoeyzyme at the phagosomal or plasma membrane (Roos *et al.*, 2003). NADPH oxidase generates superoxide by transfer of an electron to oxygen (Quinn and Gauss, 2004). Superoxide is then converted to hydrogen peroxide spontaneously or catalytically by dismutases. The azurophil granule

enzyme myeloperoxidase (MPO) uses hydrogen peroxide in turn to produce hypochlorous acid. ROS can cause significant damage to pathogenic microorganisms through the oxidation of lipids, proteins and nucleic acids (Fang, 2004). In addition, ROS can diffuse across host membranes to facilitate either inflammatory signaling events in immune cells that result in host tissue damage, or anti-inflammatory signaling events such as the induction of the transcriptional antioxidant response (Dupre-Crochet *et al.*, 2013).

1.2.3 *Mechanisms of cationic antimicrobial activity*

Interestingly, every host-derived, non-oxidative component discussed throughout this section (*1.2 Host-derived antimicrobials*) has cationic features. In general, CAPs/CAMPs have a broad spectrum of antimicrobial activity against a wide variety of pathogenic microorganisms. CAP/CAMP association with the pathogen surface is largely non-specific and is highly dependent on charge-charge interactions (Moal and Servin, 2006). Biophysical studies using membrane modeling have elucidated several mechanisms by which CAPs/CAMPs kill bacteria (Moal and Servin, 2006). Based on variations in size, amphipathicity, cationic charge and amino acid composition, CAPs/CAMPs interact with target bacterial membranes in distinct ways that fall within two general models. The 'pore-formation' model requires precise oligomerization of the CAP/CAMP followed by coordinated insertion into the bacterial membrane leading to formation of ion channels and transmembrane pores that disrupt membrane integrity and cellular homeostasis (Pazgier *et al.*, 2006; Wiesner and Vilcinskas, 2010). The 'carpet' model requires adsorption of the CAP/CAMP to the bacterial membrane in a parallel orientation. Eventually, the CAP/CAMP reaches a critical concentration on the bacterial surface causing disruption of transmembrane potential and irreversible membrane damage (Pazgier *et al.*, 2006; Wiesner and Vilcinskas, 2010). There is growing evidence that CAPs/CAMPs have additional mechanisms to disrupt bacterial integrity. CAPs/CAMPs translocate across both the inner- and outer-membrane to gain access

to the periplasm and cytoplasm, and interrupt cell division, inhibit essential enzymes, impede cell wall turnover, and reduce nucleic acid synthesis (Wiesner and Vilcinskas, 2010). Surprisingly, many CAPs/CAMPs have also been recognized for their ability to act as opsonins to enhance pathogen engulfment by innate immune cells. Opsonization by host-derived antimicrobials including azurocidin, BPI and HNPs, augments the phagocytic uptake of bacteria by monocytes, macrophages and neutrophils (Schultz, H and Weiss, 2009; Soehnlein and Lindbom, 2009).

1.3 *N. gonorrhoeae* virulence factors contributing to antimicrobial resistance

The human host has a significant arsenal of antimicrobials to combat invading pathogens. However, Gc possesses virulence factors that contribute to defense from the host immune system. In this section, I focus on Gc virulence factors that are particularly important for antimicrobial defense, but have additional defensive roles in innate immune protection.

1.3.1 *Lipooligosaccharide*

Neisseriaceae produce the natural lipopolysaccharide variant lipooligosaccharide (LOS), which lacks the O-antigen glycan polymer. LOS is composed of an inner-core glycolipid called lipid A that anchors the structure in the bacterial outer membrane. The remainder of the inner-core is composed of two 3-deoxy-D-manno-octulosonic acid (KDO) residues and two heptoses that form a scaffold for the outer-core oligosaccharide chains. Many of the glycosyl transferases responsible for extension of the oligosaccharide chains have phase-variable expression due to slipped-strand mispairing events in homopolymeric G nucleotide tracts that put the genes in and out of frame (Shafer *et al.*, 2002). Variations in the length of the oligosaccharide chains significantly impact recognition of Gc by the host innate immune system (Shafer *et al.*, 2002).

The innate immune system recognizes LOS through the cell surface toll-like receptor 4 (TLR4) in association with myeloid differentiation factor 2 (MD2), which are both expressed by epithelial cells, neutrophils, macrophages and dendritic cells (Park *et al.*, 2009). Lipid A is the conserved molecular pattern on LOS that engages the TLR4/MD2 heterodimer. Hexaacylated lipid A from Gc LOS stimulates TLR4/MD2, and causes transduction of downstream signaling events culminating in secretion of proinflammatory cytokines (John *et al.*, 2012). Gc also enzymatically modifies the lipid A portion of LOS, which influences the magnitude of TLR4/MD2 activation (John *et al.*, 2012).

1.3.1.1 *LptA*

The Gc LOS phosphoethanolamine (PEA) transferase A (LptA) catalyzes the addition of PEA to the 4' position on lipid A (Cox 2003). PEA-modified LOS induces higher proinflammatory TNF-α secretion by THP-1 monocytes compared with unmodified LOS (John *et al.*, 2012). Decreased TNF-α secretion by THP-1 monocytes is also observed in the absence of PEA-modification in an *lptA* mutant meningococcal strain (John *et al.*, 2012). In addition, LptA modification of LOS improves Gc resistance to killing by CAMPs and NHS (Lewis *et al.*, 2009; Lewis *et al.*, 2013). An *lptA* mutant of Gc strain FA19 has increased susceptibility to killing by polymyxin B and LL-37 (Lewis *et al.*, 2009), and increased sensitivity to killing by complement in normal human serum (Lewis *et al.*, 2013). The FA19 *lptA* mutant is more sensitive to complement-mediated killing, because C4-binding protein (C4BP) binds less efficiently to porin in the absence of PEA-modified LOS (Lewis *et al.*, 2013). LptA is also important *in vivo* for survival of Gc. *lptA* mutant Gc is attenuated in both experimental male infection and cervicovaginal murine challenge (Hobbs *et al.*, 2013; Packiam *et al.*, 2014). In *Chapter 3*, I report the importance of LptA for Gc defense against neutrophils and their antimicrobial components.

1.3.1.2 *Lst*

In addition to lipid A modification, Gc modifies the terminal galactose of the lacto-*N*-neotetraose (LNT) moiety on outer-core oligosaccharide of LOS with sialic acid (Shell *et al.*, 2002). Sialylation is catalyzed by the α-2,3 LOS sialyltransferase (Lst), and requires exogenous 5'-cytidinemonophospho-*N*-acetylneuraminic acid (CMP-NANA) from the host (Shell *et al.*, 2002). Sialylation of LOS blocks complement-mediated killing through binding of factor H-binding protein (fHBP), a negative regulatory factor of the alternative pathway of the complement cascade (Shell *et al.*, 2002). LOS sialylation also confers resistance to opsonic and nonopsonic phagocytosis by neutrophils, and enhances Gc survival in the murine genital tract (Wu and Jerse, 2006). Sialylated LOS additionally reduces binding of CAMPs to the Gc surface (Lewis *et al.*, 2015).

1.3.2 *Antimicrobial efflux pumps*

LOS and modifications to LOS create a physical and electrostatic barrier to impede interactions of antimicrobials with Gc. However, in many instances, antimicrobials circumvent the LOS barrier to either interact with the Gc surface or gain entry into the cell. Gc expresses four antimicrobial efflux systems that recognize and export structurally diverse toxic compounds from the cell including CAMPs, fatty acids, detergents and clinically important antibiotics (Pan and Spratt, 1994; Hagman *et al.*, 1995; Lee and Shafer, 1999; Rouquette-Loughlin *et al.*, 2003; Rouquette-Loughlin *et al.*, 2005).

1.3.2.1 *MtrCDE*

The multiple transferrable resistance (*mtr*) system encodes for a tripartite efflux pump, which belongs to the hydrophobic and amphiphilic efflux resistance-nodulation-cell division (HAE-RND) family. HAE-RND pumps are crucial in Gram-negative bacteria for efflux of antimicrobial and toxic compounds (Hagman *et al.*, 1995). Tripartite pumps are composed of inner and outer membrane channels, which

are connected through a membrane-fusion protein. The three components form a functional pump that spans the inner and outer membrane of Gram-negative bacteria, and exports harmful compounds completely out of the cell. MtrD is a proton motive force-dependent, inner-membrane channel, which connects to the MtrE outer-membrane channel through the MtrC membrane-fusion protein and assembles in a 3:6:3 stoichiometry (MtrD:MtrC:MtrE) (Lei *et al.*, 2014). Expression of MtrCDE is controlled by both a transcriptional repressor (MtrR) and an activator (MtrA) (Zalucki *et al.*, 2012).

MtrCDE facilitates efflux of a wide variety of antimicrobial compounds such as antibiotics, nonionic detergents, antimicrobial peptides, bile salts and steroidal hormones (Hagman *et al.*, 1995; Delahay *et al.*, 1997; Shafer *et al.*, 1998). Clinical isolates harboring mutations in the *mtr* system exhibit increased resistance to penicillin, macrolides, tetracycline and extended-spectrum cephalosporins (Warner *et al.*, 2008). These mutations invariably increase expression of the efflux pump, which can be favorable for Gc survival during infection. Interestingly, Mtr is also responsible for export of the host-derived CAMP LL-37, although it is unclear how LL-37 gains access into the cell (Shafer *et al.*, 1998). In addition, MtrCDE is important for *in vivo* survival of Gc. Mtr-deficient mutants are highly attenuated in the cervicovaginal murine model (Jerse *et al.*, 2003).

1.3.2.2 *FarAB*

The fatty acid resistance (FarAB) efflux pump is important for removing toxic long-chain fatty acids from Gc, and was the second efflux system identified in Gc (Lee and Shafer, 1999). FarAB expression is regulated by a transcriptional repressor (FarR), which in turn is regulated by MtrR (Lee *et al.*, 2003). The *far* system is composed of a periplasmic, membrane-fusion protein (FarA) and an inner membrane channel (FarB), and requires the MtrE outer membrane channel to form a functional pump (Lee and Shafer, 1999). However, FarAB-deficient Gc is not attenuated in the cervicovaginal murine

model (Jerse *et al.*, 2003). The *far* system may confer protection to Gc at mucosal surfaces, which are bathed with antimicrobial fatty acids (Shafer *et al.*, 1998).

1.3.2.3 *NorM*

The NorM system was identified in Gc by genome homology comparisons with *norM* from *V. parahaemolyticus* (Rouquette-Loughlin *et al.*, 2003). The NorM efflux pump is a member of the multidrug and toxic compound extrusion (MATE) family. NorM is known to export quaternary ammonium compounds and flouroquinolones, and is involved in antibiotic resistance in Gc (Rouquette-Loughlin *et al.*, 2003). NorM from *V. parahaemolyticus* and the homologue YdhE from *E. coli* have been shown to mediate resistance to cationic dyes and aminoglycosides, as well as flouroquinolones (Rouquette-Loughlin *et al.*, 2003).

1.3.2.4 *MacAB*

The Mac system was identified in Gc by genome homology comparisons with the *macA* and *macB* genes in *E. coli* (Rouquette-Loughlin *et al.*, 2005). The *mac* system is composed of a periplasmic membrane-fusion protein (MacA), and an ATP-binding inner membrane channel (MacB). As with *far*, the *mac* system requires the MtrE outer membrane channel for complete efflux of pump substrates out of the cell (Rouquette-Loughlin *et al.*, 2005). MacA-MacB-MtrE recognizes and exports macrolides, and along with MtrCDE, may contribute to decreased antibiotic susceptibility in Gc. The MacA-MacB-TolC efflux system also improves resistance of *E. coli* to macrolides (Lu and Zgurskaya, 2013).

1.4 Molecular basis for human specificity of Gc

Gc is exquisitely adapted to colonize humans, and has a strict host tropism for human infection. Gc possesses virulence factors that facilitate binding of human components to the bacterial surface, which, in turn, mediate serum resistance and nutrient acquisition in the context of its human host.

The complement system is an important part of the host innate immune response to pathogenic bacteria, and pathogenic *Neisseria* in particular, which is reflected in the sensitivity of complement-deficient individuals to invasive infection with *Neisseria* (Ram *et al.*, 2010) (Discussed further in *1.5.1 Opsonic uptake*). Sialylated LOS and porin on the Gc surface bind to fHBP, and porin binds to C4BP, which protect Gc from complement-mediated lysis in human serum (Ram *et al.*, 2001; Gulati *et al.*, 2005). Binding of both of these host factors by Gc is human-specific, as Gc does not bind either fHBP or C4BP from other species including rodents and primates (Pan *et al.*, 2014).

Nutrient acquisition is crucial to bacterial survival in the host. One of these nutrients, iron, is essential for survival of nearly all bacteria. During human infection, Gc is exposed to a highly iron-limiting environment, but requires iron for survival (Noto and Cornelissen, 2008). Gc possesses several receptors that extract iron from host iron-sequestering molecules such as transferrin, lactoferrin and hemoglobin (Noto and Cornelissen, 2008). Gc preferentially binds human transferrin and lactoferrin, but does not bind either of these proteins from bovine and porcine hosts (Pan *et al.*, 2014). Transferrin circulates in human serum, and lactoferrin is abundant in neutrophils as well as in human secretions including milk, mucus and tears (Sinha *et al.*, 2013). In addition, a transferrin receptor-null Gc mutant is comprised for survival in the human urethral challenge model of infection, which emphasizes the importance of these of these iron acquisition systems to Gc (Hobbs *et al.*, 2011).

1.5 Neutrophil phagocytosis of Gc

Gc encounters abundant neutrophils during infection. Exudates from infected individuals show Gc associated with and inside neutrophils. Uptake of Gc into neutrophils has been demonstrated to occur through both opsonic and non-opsonic mechanisms.

1.5.1 *Opsonic uptake*

Neutrophil CR3 and Fc surface receptors recognize Gc that has been opsonized with host complement and immunoglobulin, respectively (Groves *et al.*, 2008). Persons that are infected with Gc produce IgA, IgG and IgM antibodies against Gc surface structures including LOS, porin, pili and Opa proteins (Johnson and Criss, 2011). However, Gc evade B-cell humoral responses due to constant phase and antigenic variation of surface structures (Virji, 2009). Gc also express an IgA protease capable of degrading host-derived IgA at mucosal surfaces (Blake and Swanson, 1978).

Complement may be a more effective for opsonizing Gc, although Gc has several factors to resist complement deposition. Complement is an important component of innate immunity that is found in normal human serum (NHS). The complement cascade is comprised of three separate pathways, and involves over thirty proteins. The classical, alternative and lectin pathways converge on proteolytic cleavage of C3, and culminate in assembly of the membrane attack complex (Ram 2010). Gc porin and sialylated LOS bind to host C4BP and fHBP (Ram *et al.*, 2001; Gulati *et al.*, 2005). Binding of C4BP and fHBP facilitates Gc resistance to complement-mediated killing, but results in deposition of iC3b on the Gc surface. Complement-opsonized Gc are recognized by CR3 on the neutrophil surface, and are engulfed into phagosomes.

1.5.2 *Non-opsonic uptake*

In the absence of opsonization, Gc drives internalization by PMNs through Opa-mediated interactions. Gc has eleven Opa proteins that are all phase-variable due to slipped-strand mispairing events in pentameric nucleotide repeats in *opa* gene sequences, which put the genes in and out of frame (Murphy *et al.*, 1989; Dempsey *et al.*, 1991). Phase variation occurs at a rate of approximately 10^{-3} to 10^{-4} variants/cell/generation *in vitro*, and allows for any combination of zero to eleven Opa proteins to be expressed simultaneously by Gc (Mayer, 1982).

A subset of Opa proteins binds to CEACAMs on PMNs to induce internalization, and an even smaller number binds to heparin sulfate proteoglycans (HSPGs) (Sadarangani *et al.*, 2011). Only Opa proteins binding CEACAMs (Opa_{CEACAM}) have been shown to be involved in interactions with neutrophils (Sadarangani *et al.*, 2011). Neutrophils express CEACAM1, CEACAM3 and CEACAM6, which all facilitate internalization of Opa_{CEACAM} Gc. Interestingly, $Opa^{(-)}$ Gc survive better than Opa_{CEACAM} Gc following *in vitro* exposure to neutrophils (Rest *et al.*, 1982; Criss *et al.*, 2009). We have demonstrated that internalized $Opa^{(-)}$ Gc are contained in phagosomes that avoid fusion with azurophil granules (Johnson and Criss, 2013). Conversely, Opa_{CEACAM} Gc induce signaling events through CEACAM that drive Gc internalization, and subsequent residence in phagosomes containing azurophil granule contents (Johnson *et al.*, 2015). However, Opa protein expression is preferentially selected for in the male and female reproductive tracts, as well as in the murine cervicovaginal model (Swanson *et al.*, 1988; Jerse, 1999). Opa expression may be important for Gc interaction with other cell types, such as epithelial cells, that occurs prior to the recruitment of neutrophils.

Our lab has shown that adherent, chemokine-treated neutrophils can internalize non-opsonized, $Opa^{(-)}$ Gc (Criss *et al.*, 2009). Opsonin-independent, Opa-independent phagocytosis by neutrophils could occur in two ways. First, Gc pilin and porin have been shown to interact cooperatively with CR3 on cervical epithelial cells (Edwards *et al.*, 2002), and we have preliminary evidence that CR3 contributes to phagocytosis of non-opsonized Gc by human neutrophils (A. Smirnov, K. Daily, B. Johnson, and A. Criss, manuscript in preparation). Second, *N. meningitidis* with the lacto-N-neotetraose (LNnT) LOS moiety are phagocytosed by neutrophils through an opsonin-independent mechanism (Estabrook *et al.*, 1998), although it is not yet known if Gc with LNnT LOS are similarly internalized.

1.6 Neutrophil extracellular traps

Non-phagocytosed Gc are left to contend with neutrophil extracellular killing mechanisms. Recently, the formation of neutrophil extracellular traps (NETs) was observed as part of the host innate immune response to infection (Brinkmann *et al.*, 2004). NET formation occurs when neutrophils undergo a unique form of cell death called NETosis that is morphologically different from necrosis and apoptosis (Fuchs *et al.*, 2007). NETosis is defined by prelytic decondesation of chromatin, and disruption of the nuclear membrane (Fuchs *et al.*, 2007). Following breakdown of the nuclear membrane, DNA and histones mix with granule components, and are released extracellularly as web-like fibers (Fuchs *et al.*, 2007). There is also growing evidence suggesting that NETosis does not always culminate in neutrophil cell death. Viable neutrophils can form NETs through vesicular release of mitochondrial DNA at the plasma membrane (Yousefi *et al.*, 2009). NETs have been shown to have antimicrobial activity against bacteria, viruses and parasites (Brinkmann *et al.*, 2004). NET structures are also important for immobilization and growth inhibition of pathogens (Brinkmann *et al.*, 2004).

1.7 *N. gonorrhoeae* defenses against killing by neutrophils

A hallmark of gonorrhea is the abundant influx of neutrophils to the site of infection. Neutrophils mount a robust immune response that creates a toxic, unfavorable environment for many pathogenic microorganisms. However, Gc resists clearance by neutrophils due to the contribution of key virulence factors. In this section, I will discuss how Gc defends against the oxidative and non-oxidative arms of the neutrophil antimicrobial arsenal.

1.7.1 *Oxidative defense*

Activated neutrophils produce an oxidative burst that relies on initial production of superoxide by NADPH oxidase, which assembles at the plasma or phagocytic membrane (Discussed in *1.2.2.8*

Reactive oxygen species). Oxidative enzymes successively convert superoxide → hydrogen peroxide → hypochlorous acid. ROS induce oxidative damage to bacterial proteins, lipids and DNA. However, Gc has several mechanisms to mitigate oxidative damage including suppression of the oxidative burst, detoxification of ROS, repair of oxidative damage and transcriptional upregulation of antioxidant gene expression.

1.7.1.1 *Suppression of oxidative burst*

Opa$^{(-)}$ Gc do not stimulate an oxidative burst by primary human neutrophils (Rest *et al.*, 1982; Criss and Seifert, 2008). The burst produced by neutrophil stimulation with Opa^{+} Gc is also significantly lower than stimulation with other bacteria including serum-opsonized *S. aureus* and with chemical stimuli such as phorbol-12-myristate-13-acetate (PMA) (Simons *et al.*, 2005; Criss and Seifert, 2008). Gc stem the oxidative burst in several ways. Gc senses lactate released by neutrophils, and increases oxygen consumption to limit the availability of NADPH oxidase substrate. Purified Gc porin also suppresses the oxidative burst generated by stimulation with Opa^{+} Gc (Lorenzen *et al.*, 2000). In addition, Opa$^{(-)}$ Gc interferes with assembly of NADPH oxidase, suppresses the burst generated by stimulation with serum-opsonized *S. aureus* and methionylleucylphenylalanine (fMLP), and requires active protein synthesis and Gc cell contact with neutrophils (Criss and Seifert, 2008; Smirnov *et al.*, 2014).

1.7.1.2 *Detoxification of ROS*

Gc possesses enzymes to directly detoxify ROS. Superoxide dismutase B (SodB) catalyzes conversion of superoxide to hydrogen peroxide. An abundant catalase (KatA) in the Gc cytoplasm rapidly breaks down toxic hydrogen peroxide to water and oxygen. KatA-deficient Gc are significantly more sensitive *in vitro* to superoxide and hydrogen peroxide exposure (Soler-García and Jerse, 2004; Stohl *et al.*, 2005). The cytochrome c peroxidase (*ccp*) is also involved in ROS defense in Gc, as a *ccp* mutant is

more senstive to killing by hydorgen peroxide (Turner and McGivan, 2003). In addition, the MntABC manganese transporter imports exogenous Mn(II), which can nonezymatically scavenge superoxide and hydrogen peroxide (Seib *et al.*, 2006). Glutathione is another important ROS scavenger, which is maintained in its reduced state by Gor, a Gc glutathione reductase (Seib *et al.*, 2006).

1.7.1.3 *Repair of oxidative damage*

If ROS are not quickly detoxified, they can significantly damage bacterial components. Two methionine sulfoxide reductases (MsrA and MsrB) repair oxidative damage to methoinine residues in affected proteins. Gc also expresses enzymes involved in DNA base and nucleotide excision repair, as well as in recombination repair, including RecN, that contribute oxidative repair of nucleic acid (Stohl and Seifert, 2006). Additionally, the Ngo1686 metalloprotease contributes to Gc protection from hydrogen peroxide and the lipid oxidant cumene hydrogen peroxide (Stohl *et al.*, 2005; Stohl *et al.*, 2013). In addition, Ngo1686 and RecN are important for Gc survival during exposure to neutrophils (Stohl *et al.*, 2005; Criss *et al.*, 2009).

1.7.1.4 *Transcriptional regulation during oxidative stress*

Gc exposed to oxidative stress conditions upregulate the expression of protective factors. Preincubating Gc with sublethal concentrations of hydrogen peroxide improves their survival during exposure to neutrophils (Criss *et al.*, 2009), which suggests that the Gc protective response to oxidative stress also mediates protection against neutrophil killing. A study using microarray analysis to detect changes in gene expression following hydrogen peroxide exposure found that the antioxidant genes *recN* and *ngo1686* were upregulated (Stohl *et al.*, 2005).

1.7.1.5 *Role of neutrophil ROS during a Gc infection*

Surprisingly, despite the numerous mechanisms Gc has for oxidative protection, they do not appear to play a significant role in Gc defense against neutrophils. Mutants strains of Gc deficient for KatA, SodB or MntABC are not more sensitive to neutrophil killing (Seib *et al.*, 2005; Criss *et al.*, 2009). ROS-deficient neutrophils from chronic granulomatous disease individuals and neutrophils in anoxic conditions have similar antigonococcal activity to normal neutrophils in favorable conditions for ROS production (Rest *et al.*, 1982; Casey *et al.*, 1986; Criss and Seifert, 2008). We have also shown that Gc survival, including survival of *recN* and *ngo1686* mutant strains, is not improved in the presence of neutrophils that are preteated with the NADPH oxidase inhibitor diphenylidene iodonium (DPI). Additionally, Opa^+ Gc that stimulate an oxidative burst do not survive better in the presence of DPI-treated neutrophils (Criss *et al.*, 2009). Taken together, these observations suggest that neutrophil antigonococcal activity is primarily non-oxidative.

1.7.2 *Non-oxidative defense*

Neutrophils have a robust non-oxidative arsenal. Oxidative-independent neutrophil killing mechanisms were beginning to be characterized in the 1970s, and several groups identified non-oxidative components with cationic and proteolytic antimicrobial activities stored in cytoplasmic granules (Rest, 1979; Shafer *et al.*, 1984; W.M. Shafer, Onunka, *et al.*, 1986). During infection, activated neutrophils mobilize antimicrobial-containing granules to fuse internally with phagosomes or to fuse at the plasma membrane. Pathogens contained within phagosomes become overwhelmed with increasing antimicrobial concentrations as more and more granules fuse to release their contents. Extracellular pathogens are also exposed to degranulated antimicrobial contents, but at significantly lower concentrations. In many instances, pathogens induce NET release by neutrophils. NETs immobilize pathogens, allowing granule components that decorate the web-like structure to kill trapped

microorganisms. However, Gc employs virulence factors during neutrophil exposure that impede binding and killing by cationic antimicrobials, remove toxic components from the bacterial cell by antimicrobial efflux, and enable survival within and escape from NET structures.

1.7.2.1 *Protection against binding and killing by cationic antimicrobials*

LOS and modifications of LOS (discussed in section *1.3.1 Lipooligosaccharide*) physically and electrostatically impede CAP/CAMP interactions with the Gc surface. LOS modifications have important roles in Gc resistance to killing by CAMPs (W.M. Shafer, Onunka, *et al.*, 1986; Shafer *et al.*, 1998; Lewis *et al.*, 2015). In particular, LptA modification improves Gc survival during neutrophil exposure, and directly contributes to Gc resistance to several neutrophil components including CAP37/Azurocidin, HNP-1 and cathepsin G (discussed in *Chapter 3*) (Handing and Criss, 2015). In addition, sialylation of LOS decreases binding of cathepsin G to the Gc surface (W.M. Shafer, Onunka, *et al.*, 1986).

1.7.2.2 *Antimicrobial efflux*

Gc has four known antimicrobial efflux pumps that export structurally diverse substrates, which include host-derived components such as toxic fatty acids (FAs) and CAMPs (discussed in section *1.3.2 Antimicrobial efflux pumps*) (Pan and Spratt, 1994; Hagman *et al.*, 1995; Lee and Shafer, 1999; Rouquette-Loughlin *et al.*, 2003; Rouquette-Loughlin *et al.*, 2005). The *far* system recognizes and removes harmful FAs from the bacterial cell (Lee and Shafer, 1999). Gc may encounter toxic FAs during infection, although no specific role for the pump has been described for Gc defense against host innate immunity. The *mtr* system enables export of a wide variety of antimicrobial compounds including the CAMP LL-37 (Shafer *et al.*, 1998). As I will discuss in *chapter 4*, MtrCDE also protects Gc from extracellular killing, which is in part due to export of degranulated neutrophil antimicrobials by the efflux pump.

1.7.2.3 *Survival within and escape from NETs*

The formation of NETs is an important element of the host innate immune response to bacterial pathogens (Brinkmann *et al.*, 2004). We and others have shown that Gc induces NET release *in vitro*, but is not susceptible to NET-mediated killing (Gunderson and Seifert, 2015; Handing and Criss, 2015; Juneau *et al.*, 2015). Gc expresses a thermonuclease (Nuc) that degrades the NET DNA backbone to facilitate Gc escape (Juneau *et al.*, 2015). Freed Gc are no longer exposed to the high local concentrations of antimicrobial granule components found in NET structures. Additionally, we have found that LptA is an important factor for protecting Gc during exposure to NETs, which has previously been reported for *N. meningitidis* (discussed in *Chapter 3*) (Lappann *et al.*, 2013; Handing and Criss, 2015).

1.8 Dissertation goals and significance

Despite the rise in the worldwide incidence of gonorrhea, and the high frequency of multidrug resistance to every current and past antibiotic recommended for treatment, we have an incomplete understanding of Gc pathogenesis in the obligate human host. Researchers are working to address fundamental gaps in our knowledge of the infection process, but the questions far outnumber the answers. The goal of my thesis research is to evaluate the roles of Gc virulence factors in relation to antimicrobial defense and in interactions with neutrophils. In Chapter 2, I investigate the antigonococcal activity of β-defensin 22, one epithelium-derived component produced by the mucosal epithelium in the reproductive tract, as a potential new therapeutic avenue for combating Gc. In Chapter 3, I examine the impact of LOS modification by LptA for Gc defense against neutrophil-derived antimicrobials, and for interactions with neutrophils. In Chapter 4, I explore the role of the MtrCDE efflux pump during exposure to neutrophils, and assess its importance for export of neutrophil-derived antimicrobials. Since Gc adeptly evades the adaptive immune response, a keen understanding of the

innate immune response during gonococcal infection is essential to developing therapeutic solutions to combat Gc.

1.9 Figures

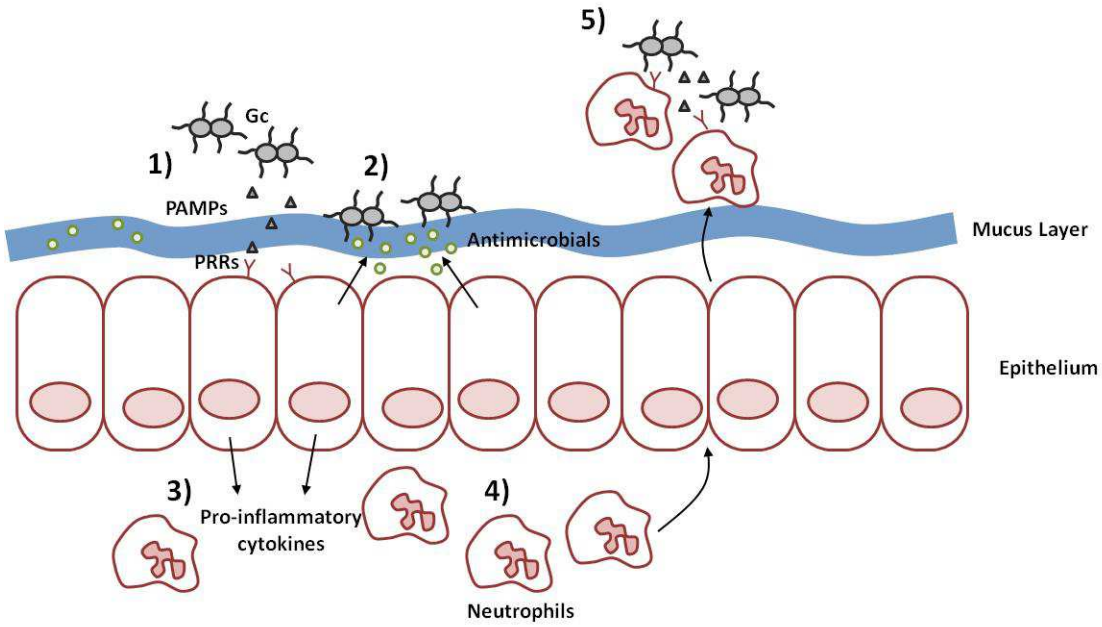

Figure 1. Gc encounters the mucosal epithelium.

1) Gc sheds PAMPs that are recognized by PRRs on the epithelial cell surface. Activated PRRs signal epithelial cells to upregulate expression and release of additional 2) antimicrobials to combat Gc and 3) proinflammatory cytokines to alert the host immune system. 4) The cytokine gradient favors recruitment of neutrophils to the site of infection. 5) Neutrophils transmigrate across the epithelium, become activated and mount a robust antimicrobial response to control Gc numbers.

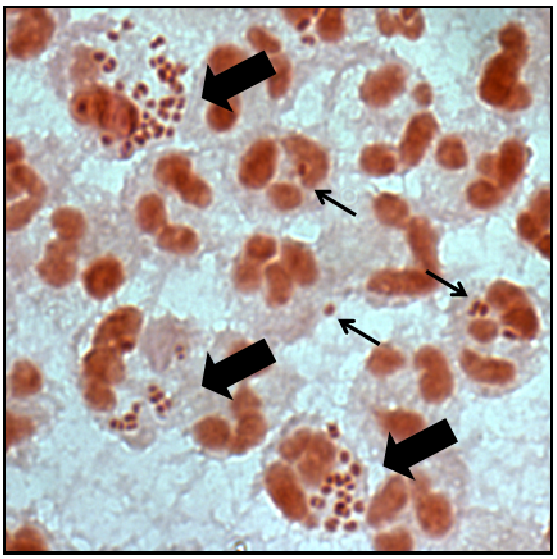

Figure 2. Gonorrheal exudates contain numerous PMNs with associated Gc.

Gram stain of the urethral exudate from a male with uncomplicated gonorrhea. Some PMNs associate with single diplococci (thin arrow), while others have multiple adherent and internalized Gc (thick arrow). Note that the majority of PMNs in the exudate are uninfected. From Johnson and Criss *Front. Microbiol.*, 2:77, 2011. doi:10.3389/fmicb.2011.00077 with permission per guidelines.

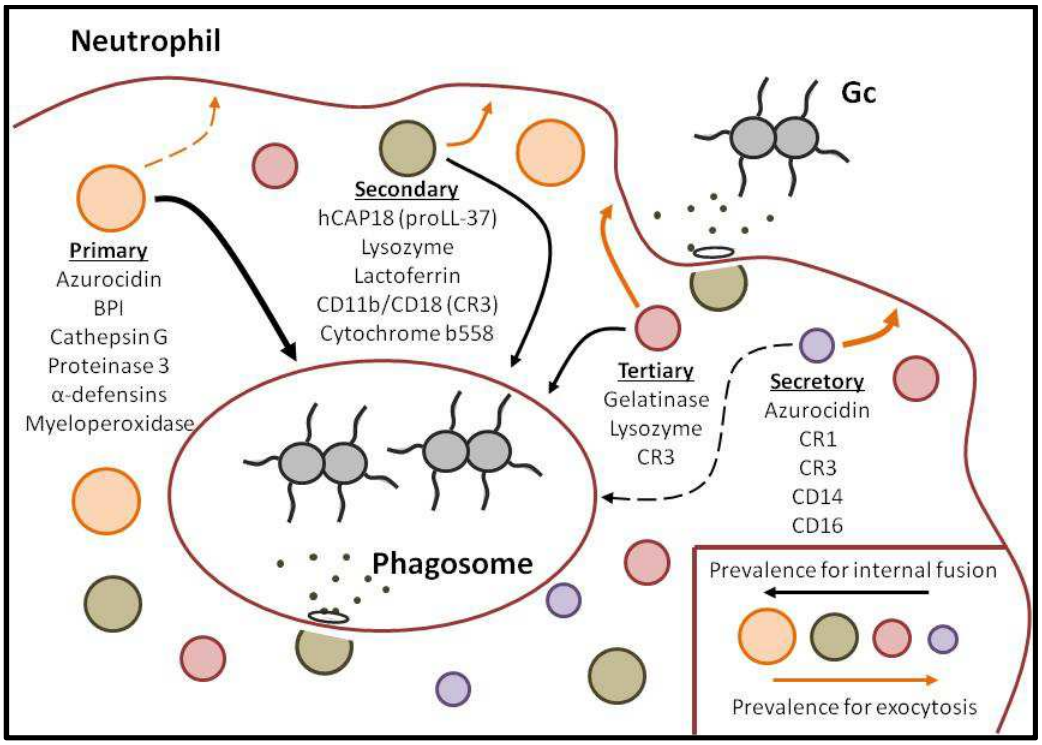

Figure 3. Neutrophil granule content.

List of components in each granule subset and indication of preference for internal fusion (black arrows) versus exocytosis (orange arrows). Adapted from **Pham, C.** *Nature Reviews Immunology.* 6(7): 541-550, 2006.

2. Chapter 2: Antigonococcal activity of β-defensin 22

2.1 Introduction

Neisseria gonorrhoeae (Gc) is a gram-negative diplococcus and the etiological agent of the sexually-transmitted infection (STI) gonorrhea. In recent years, the estimated number of annual cases of the disease has surpassed 106 million (World Health Organization, 2011; World Health Organization, 2012). The US Centers for Disease Control and Prevention has categorized Gc as an urgent global health threat due to widespread emergence of multidrug-resistant clinical isolates (CDC, 2011; CDC, 2013). Infection with Gc commonly presents as cervicitis in females and urethritis is males. Failure to treat the disease can result in secondary complications including pelvic inflammatory disease and ectopic pregnancy in women and sterility in both men and women.

Transmission of gonorrhea brings Gc into contact with host mucosal surfaces. Mucosal epithelial cells lining the genitourinary tract release β-defensins (βDs) and other antimicrobials to combat infectious agents (Patil *et al.*, 2005). βDs are cysteine-rich cationic peptides that have broad-spectrum antimicrobial activity against bacteria, enveloped viruses and fungi (Liu *et al.*, 2013). The vast majority of βDs are preferentially expressed in the male reproductive tract with minimal to no expression in other tissues (Patil *et al.*, 2005; Yudin *et al.*, 2008). βD expression is region-specific, and is primarily concentrated in the epididymis and testis (Rao, 2002; Patil *et al.*, 2005). βDs are integral in innate immune defense from infection (Patil *et al.*, 2005). βDs are also actively involved in reproductive processes, and enhance sperm maturation and motility (Patil *et al.*, 2005). Their expression is regulated by androgens, and they are secreted into luminal plasma and bind to maturing, but not immature, sperm (Patil *et al.*, 2005).

β-defensin 22 (βD22, also called βD126 or DEFB126 in humans, epididymal secretory protein 13.2 in macaques, and E-3 in rats) is a secreted glycoprotein member of the βD family. βD22 orthologs have

also been identified in mouse and primates (Yudin *et al.*, 2008; Tollner *et al.*, 2012). Human βD126 and rat βD22 share 45% identity (E-value $4E^{-18}$; NCBI Blast). βD22 is secreted in the epididymis and coats the sperm glycocalyx, an immunoprotective scaffold composed of surface-associated oligosaccharides (Rao, 2002; Zanich, 2003; Tollner *et al.*, 2012). The expression pattern of βD22 and its ability to coat sperm are maintained across species (Tollner *et al.*, 2012). βD22 possesses an N-terminal signal sequence, a defensin β2 superfamily motif (Dβ2 motif), and a C-terminal lectin-like motif (Figure 4A). The defensin motif of βD22 contains the six conserved cysteine residues that are characteristic for β-defensins (Figure 4B). The lectin-like motif is glycosylated and contributes to the glycocalyx of sperm (Tollner *et al.*, 2012). βD22 facilitates attachment of sperm to the oviductal epithelium, enhances capacitation and sperm-egg interactions within the female reproductive tract, and protects sperm from immune recognition (Yudin *et al.*, 2008; Tollner *et al.*, 2012). The β-defensin domain of human and rat βD22 has been shown to have antimicrobial activity against the fungus *Candida albicans,* the gram-negative bacterium *E. coli,* and the gram-positive bacterium *Staphylococcus aureus* (Diao *et al.*, 2011; Liu *et al.*, 2013). βD22 is preferentially expressed in the reproductive tract of the host (Tollner *et al.*, 2012). This site is a prominent environmental niche for *Neisseria gonorrhoeae* and other sexually transmitted pathogens. However, prior to this study, the antimicrobial activity of βD22 against these pathogens had not been explored.

Here, we report that *Neisseria gonorrhoeae* is sensitive to killing by a recombinant form of rat βD22, and that killing is both concentration-dependent and time-dependent. We show that in addition to commonly used Gc laboratory strains, βD22 has activity against several “superbug” antibiotic-resistant isolates. Lastly, we demonstrate that βD22 interacts with and coats the Gc surface. We provide evidence that the lectin-like domain of βD22 mediates cell-surface association while the defensin-like domain mediates bacterial killing, suggesting a two-step model for βD22 antimicrobial activity.

2.2 Materials and Methods

2.2.1 *Bacterial strains*

The Gc laboratory strains used were a constitutively piliated, opacity protein-deficient derivative of strain FA1090 (Ball and Criss, 2013), MS11 VD300, which is predominantly piliated and phenotypically Opa-negative (from Dr. H. Steven Seifert, Northwestern University), as well as clinical strains FA6140, F89 and 35/02 (from Dr. Robert Nicholas, UNC Chapel Hill). Gc were grown on gonococcal medium base (Difco) plus Kellogg's supplements (Kellogg *et al.*, 1963) for 20 hours at 37˚C in 5% CO_2.

2.2.2 *Sequence comparison*

Rat βD22 (AF329091), human βD22 (NM_030931) and human βD2 (AF040153.1) sequences were acquired from the NCBI database. The NCBI BLAST tool (Madden, 2002)was used to elucidate sequence motifs and evaluate identity between rat and human βD22. Sequence alignment was performed using the Clustal Omega multiple sequence alignment tool (Sievers *et al.*, 2011; McWilliam *et al.*, 2013; Li *et al.*, 2015).

2.2.3 *The expression and purification of rat recombinant β-defensin 22 (rβD22) protein*

The cloning and expression of rβD22 protein in *Escherichia coli* has been previously described (Rao, 2002). *E. coli* was transformed with a pET28b-βD22-6His plasmid to express rβD22 with a 6His-tag at the C-terminus. His-tagged recombinant protein was purified from the transformed *E. coli*, using a BD TALON-immobilized, metal-affinity chromatography (IMAC) resin, as described previously (Rao, 2002). His-tagged rβD22 was eluted with imidazole and each fraction was analyzed with Coomassie Brilliant Blue R-250 staining on an SDS-PAGE gel. The 16 kDa purified His-tagged rβD22 was quantified by a Bradford assay. Fractions having the 16 kDa protein were subjected to the affinity purification protocol a

second time, and the purity of the eluted protein was reassessed as above. Eluted fractions with a single 16 kDa band were pooled and subjected to Western blot analysis using nickel-nitrilotriacetic acid conjugated to horseradish peroxidase (Ni-NTA HRP) (Qiagen) (Rao, 2002). The purified His-tagged rβD22 protein was quantified by Bradford assay and protein aliquots were stored in phosphate buffer (pH 7.2) at -80°C until use.

2.2.4 *Peptide synthesis and purification*

The defensin-like domain of rat rβD22 ($\beta D22_{DD}$; amino acid sequence "WYVRKCANKLGTCRKTCRKGEYQTDPATGKCSIGKLCCIL") and lectin-like domain of rat rβD22, with addition of 6His tag at the C-terminus ($\beta D22_{LD}$; amino acid sequence "DLKLAGQCGGADGNQAAAGTQAAGGTRAAGGTQGTGGTGATGAAATTAAPHHHHHH") were synthesized by Peptide 2.0 (Chantilly, VA), using standard fluorenylmethoxycarbonyl (Fmoc) solid-phase on a preloaded Wang resin. Fmoc-peptide-resin was cleaved using 20% piperidine in dimethylformamide (DMF) for 10 min and repeated. The crude peptide was cleaved from the resin, precipitated with cold diethyl ether, and purified by preparative reverse-phase-HPLC. The peptides were eluted using solvent A (0.1% TFA in CH_3CN) and solvent B (0.1% TFA in H_2O) at a flow rate of 1.0ml/min in a gradient. The peptide was detected with a UV detector at 220 nm. The peak with purified peptide was collected and lyophilized. The peptide was analyzed by mass spectrometry (ESI), and the peptide purity was measured by analytical HPLC.

His-tagged *Sinorhizobium meliloti* SMa1515 (accession number NP_436070.2) was used as a negative-control peptide in βD22 bacterial binding assays. *Sinorhizobium meliloti sma1515* was cloned into pSGC-His plasmid, expressed by IPTG induction and purified on a nickel-nitrilotriacetic acid (Ni-NTA) column. Peptide sequence: MSEDAFNMSIRKFLKEVGVTSQREIEETVRKGQIDGNKLKVRMTLTAEGT

DLNHVVAGEIELPHHHHHH. Purified peptide was sourced from the New York Structural Genomics Research Consortium <http://kiemlicz.med.virginia.edu/nysgrc/space_tree/view/020974>

2.2.5 *Antimicrobial assays*

Gc was resuspended at a concentration of 5 x 10^5 cfu/ml in low ionic-strength liquid media (0.2x GCBL, unsupplemented) to optimize defensin cationic conditions. Full-length rat βD22, $\beta D22_{DD}$, $\beta D22_{LD}$ and human β-defensin 2 (hβD2) (Phoenix Pharmaceuticals) were suspended in 0.2x phosphate buffer (PB; pH 7.2) and 20 µl of defensin was incubated with 180 µl of Gc for 1.5 or 3 hours at 37˚C in 5% CO_2. Gc survival for each condition was quantified by serial dilution and CFU enumeration after overnight growth on GCB. Gc survival is expressed relative to bacteria in the untreated control, which is normalized to 100% for each independent experimental condition.

2.2.6 *βD22 bacterial binding assay*

Gc were resuspended in GCB liquid medium at a concentration of 1 x 10^6 cfu/ml and allowed to adhere to plastic coverslips for 30 minutes at 37˚C in 5% CO_2. Media was aspirated, replaced with 0.2x phosphate buffer (pH 7.2) containing 200 µg/ml His-tagged βD22, $\beta D22_{LD}$, or the *S. meliloti* control peptide and incubated for 1 hour at 37˚C in 5% CO_2. Gc was fixed in 4% paraformaldehyde in PBS for 20 minutes, blocked in PBS with 1% BSA, and incubated with an anti-His-tag antibody (His.H8 mouse; Thermo Fisher) and anti-Gc antibody (rabbit; Biosource) in PBS with 1% BSA for 1 hour. Gc were then incubated with anti-mouse conjugated Alexa Fluor 555 (Life Technologies) and anti-rabbit conjugated Alexa Fluor 488 (Life Technologies) secondary antibodies for 1 hour. Images were acquired using a Nikon Eclipse E800 UV/visible fluorescence microscope with a Hamamatsu Orca-ER digital camera using Openlab software. Images were processed using Adobe Photoshop CS6 software.

2.2.7 *Statistics*

Values are the mean ± the standard error of at least three, independent replicates performed on different days. Significance was assessed using a two-tailed Student's *t*-test. A *P*-value of <0.05 was considered significant.

2.3 Results

2.3.1 *β-defensin 22 has antimicrobial activity against N. gonorrhoeae*

We first ascertained if Gc had susceptibility to βD22, since Gc is reported to be highly resistant to defensins (Qu *et al.*, 1996; Johnson *et al.*, 2015). Given the strong structural and sequence similarities of βD22 between species, we used a recombinant form of rat βD22 (rβD22), which is composed of residues 22-111 with the addition of a C-terminal His-tag (Rao, 2002). FA1090 Gc was incubated with rβD22 or human β-defensin 2 (hβD2), or in media alone. Gc colony-forming units (CFU) were enumerated after incubation, and survival was calculated as the percent bacterial survival relative to CFU of the inoculum. Exposure to 20 μg/ml rβD22 reduced Gc survival to 40% at 1.5 hours and to 25% at 3 hours. In contrast, there was no decline in bacterial survival when Gc was incubated with the same concentration of hβD2 for the same times (Figure 5A). FA1090 Gc was then incubated with increasing concentrations of rβD22 for 3 hours. Gc survival decreased in a concentration-dependent manner after exposure to rβD22, but no loss of bacterial survival was observed despite exposure to 200 μg/ml hβD2 (Figure 5B).

rβD22 antimicrobial activity was tested against commonly used Gc laboratory strains (FA1090, MS11) and multidrug-resistant "superbug" clinical isolates (FA6140, F89 and 35/02) (Figure 6). All strains of Gc tested were sensitive to killing by 20 μg/ml rβD22 for 3 hours (Figure 6). Survival ranged from 32% for strain F62 to 68% for strain MS11. Notably, all of the "superbug" strains exhibited significantly reduced survival, when compared with survival in media alone, after exposure to rβD22 that was

comparable to strain FA1090. This observation suggests that antibiotic-resistance mutations in multidrug-resistant isolates do not have any effect on bacterial susceptibility to βD22. Taken together, these results show that βD22 has modest antimicrobial activity against multiple Gc strains, including those resistant to multiple antibiotics.

2.3.2 *The defensin-like domain is responsible for the antigonococcal activity of β-defensin 22*

We next sought to determine which domain of βD22 conferred antimicrobial activity against Gc. To address this question, we recombinantly produced two peptides: the defensin domain of rβD22 (aa 22-61; $\beta D22_{DD}$) and the rβD22 lectin domain (aa 62-111; $\beta D22_{LD}$) (see Figure 4A). Gc survival was measured after bacterial exposure to full-length rβD22, $\beta D22_{DD}$, or $\beta D22_{LD}$ for 3 hours. Gc exhibited comparable susceptibility to $\beta D22_{DD}$ and to full-length βD22, including at approximately equimolar concentrations (Figure 7). However, $\beta D22_{LD}$ had no antimicrobial activity against Gc (Figure 7). These results indicate that the defensin domain of βD22 is necessary and sufficient for antigonococcal activity, in agreement with previous findings in *E. coli* and *C. albicans* (Diao *et al.*, 2011; Liu *et al.*, 2013). Moreover, these findings demonstrate that the lectin domain itself lacks antimicrobial activity against Gc.

2.3.3 *β-defensin 22 interacts with the surface of N. gonorrhoeae through its lectin domain*

βD22 is distinctive among the β-defensins for having an extended C-terminal tail containing a lectin-like domain in addition to the canonical defensin motif (Rao, 2002; Liu *et al.*, 2013). The βD22 lectin domain has been shown to bind the carbohydrate heparin and coat the sperm glycocalyx, which is composed primarily of surface-associated oligosaccharides (Diao *et al.*, 2011; Liu *et al.*, 2013). Given the antimicrobial activity of βD22 against Gc, we hypothesized that the lectin domain of βD22 interacts with the Gc surface, which is largely composed of lipooligosaccharide (LOS) and also has glycosylated proteins. To test this hypothesis, FA1090 Gc was grown to mid-logarithmic phase, then incubated with

rβD22, the lectin domain of rβD22 alone with a C-terminal His tag (rβD22$_{LD}$), or a control His-tagged peptide for 1 hour. Bacteria were washed and fixed with paraformaldehyde. Bacteria were detected with a Gc-specific antibody followed by green-fluorescent secondary antibody, while any peptides bound to the bacteria were detected with an anti-His-tag antibody followed by red-fluorescent secondary antibody. We observed a strong fluorescent signal for βD22 and βD22$_{LD}$ in association with the Gc surface, while comparably little control peptide staining was visible in proximity to Gc (Figure 8). These results demonstrate that rβD22 can directly interact with Gc, and the lectin domain is sufficient to mediate this interaction. These findings suggest that in addition to sperm, βD22 may interact with bacteria in the reproductive tract, helping to protect the host from infection with sexually transmitted agents such as Gc.

2.4 Discussion

Human and rat genomes encode approximately 40 β-defensins, and over half of these are predominantly expressed in the male reproductive tract (Patil *et al.*, 2005; Pazgier *et al.*, 2006). The abundance and variety of β-defensins suggest that these cationic peptides are positioned to protect the host against the threat of a reproductive tract infection. In this study, we investigated the antimicrobial activity of one of these defensins, β-defensin 22, against Gc. βD22 has been shown to be active against prokaryotic and eukaryotic pathogens (Diao *et al.*, 2011; Liu *et al.*, 2013). However, prior to this study, its activity against pathogens causing STIs had not been examined. We show that Gc is susceptible to killing by βD22 and that βD22 is able to associate with the Gc surface, with the lectin domain being sufficient for this interaction. Gc is reported to be highly resistant to killing by defensins (Qu *et al.*, 1996; Shafer *et al.*, 1998). To our knowledge, βD22 is the first defensin reported to be active against Gc.

Gonorrhea is a growing threat to global health, which is in large part due to the spread of multidrug resistant clinical isolates. In 2013, the CDC identified Gc as an urgent threat to public health,

due to the dramatic rise in “superbug” isolates exhibiting resistance to the latest generation of extended-spectrum cephalosporins, the last recommended line of treatment for the disease. We show that βD22 has broad antimicrobial activity at low micromolar concentrations (2.3 μM or 20 μg/ml) against all the strains tested, including three multidrug-resistant clinical isolates. The F89 and 35/02 isolates exhibit chromosomally mediated penicillin resistance and resistance to cefixime and ceftriaxone (Unemo *et al.*, 2012; Abrams *et al.*, 2015). The FA6140 isolate exhibits chromosomally mediated resistance to penicillin, tetracycline and azithromycin. It is striking that the mechanisms conferring resistance to multiple antibiotics in these “superbug” isolates (F89 and 35/02) do not protect the bacteria from killing by βD22. The resistance determinants contributing to antibiotic resistance for Gc are active areas of investigation (Tomberg *et al.*, 2013; Unemo and Shafer, 2014). Many of the determinants are novel variants of penicillin-binding protein 2 (PenA). Other mutations confer increased expression of the multiple-transferrable-resistance (Mtr) efflux system repressor (MtrR) and changes to the major porin (PorB1b). Notably, the strains tested in this study vary in their expression of outer membrane components including pili, porin, and lipooligosaccharide, as well as expression of efflux pumps, yet all exhibit some susceptibility to killing by βD22, implying a conserved target for βD22 activity. Of the strains examined, MS11 exhibited the least sensitivity to killing by βD22. MS11 carries a mutation in the *mtrR* repressor that leads to increased expression of the Mtr efflux pump, but the fact that “superbug” isolates (F89, 35/02) that carry the same *mtrR* mutation are not more resistant to βD22 implies that Mtr is not a major contributor to the increased resistance of MS11 to βD22. MS11 may have other changes to its cell envelope that confer resistance to βD22. Alternatively, βD22 may interact less efficiently with the MS11 surface and thus have reduced antimicrobial activity.

The βD22 defensin domain contains the six canonical cysteine residues that are conserved across β-defensins. Beyond this, β-defensins within species have highly dissimilar protein sequences (Rao, 2002; Patil *et al.*, 2005). Notably, we found that the antimicrobial activity of βD22 is localized to its

defensin domain, as reported for other gram-negative bacteria and fungi (Diao *et al.*, 2011; Liu *et al.*, 2013). The percent identity between βD22 and a canonical β-defensin, βD2, is low (20%). In this light, it is not surprising that Gc are sensitive to βD22 yet highly resistant to hβD2, as we previously reported (Johnson *et al.*, 2015). This suggests that there are variations in the antimicrobial activities of the defensins, which could be explained by the significant differences at the protein sequence level.

βD22 also differs from most characterized β-defensins by possessing an extended C-terminal tail containing a lectin-like motif (Rao, 2002). The βD22 lectin domain has been shown to bind carbohydrate moieties including heparin and sperm-associated oligosaccharides (Diao *et al.*, 2011; Liu *et al.*, 2013). We observed that βD22 can associate with the Gc surface, and the lectin domain alone is sufficient for mediating this interaction. The ligand on the bacterial surface for the lectin domain is not currently known. Sugars on LOS are one potential candidate, as is outer membrane or surface-exposed glycoproteins. We were unable to assess binding of the βD22 defensin domain to the Gc surface, because the recombinant construct lacks an epitope tag and there are no antibodies available against βD22. However, the defensin domain alone has antimicrobial activity against Gc, implying that in our *in vitro* conditions this peptide can interact with the bacteria. Human βD22 has been shown to neutralize soluble LPS and diminish LPS-induced release of inflammatory cytokines from RAW264.7 murine macrophages; notably, these effects were mediated by the defensin domain of βD22 , not the lectin domain (Liu *et al.*, 2013). Whether the defensin domain of βD22 can also interact with LPS at the surface of a microbe is unknown.

There is growing evidence that β-defensins expressed in the urogenital tract have dual functionality, to mediate critical reproductive events and to protect the reproductive environment from invading pathogens. βD22 is preferentially expressed in a prominent environmental niche for Gc colonization. We propose the following two-step model for βD22 antigonococcal activity. The lectin

domain first associates with carbohydrate moieties on the Gc surface to bring the peptide into close proximity to the Gc outer membrane. The defensin domain then interacts with the Gc membrane to mediate bacterial killing. There are two proposed models describing how defensins interact with bacterial membranes (Pazgier *et al.*, 2006). The 'pore formation' model requires precise peptide oligomerization for insertion into the membrane, which has not been observed for β-defensins (Pazgier *et al.*, 2006). The 'carpet' model is less structurally restricted. In this model, defensin associates with the bacterial surface, neutralizes negatively charged lipid moieties on the outer membrane and disrupts the transmembrane electrical potential. Crystallographic models using hβD2 suggest that β-defensins organize into higher-order oligomers that are stabilized by interactions between hydrophobic regions, and these are more structurally in agreement with the 'carpet' model of bacterial membrane interaction (Pazgier *et al.*, 2006). However, Gc is resistant to killing by hβD2, which could indicate that the 'pore formation' model is more likely for mediating the antigonococcal activity of βD22. It is probable that during gonorrheal infection Gc encounters epithelium-derived βD22 at mucosal surfaces lining the male reproductive tract. Interestingly, none of the strains we tested, including the clinical isolates, have gained βD22 resistance, despite the fact that β-defensins are ancient antimicrobial peptides to which Gc has likely been exposed for its entire evolutionary history with humans. Thus βD22 is an attractive candidate for development as a new antigonococcal therapeutic, which is urgently needed given the looming threat of untreatable gonorrhea.

2.5 Figures

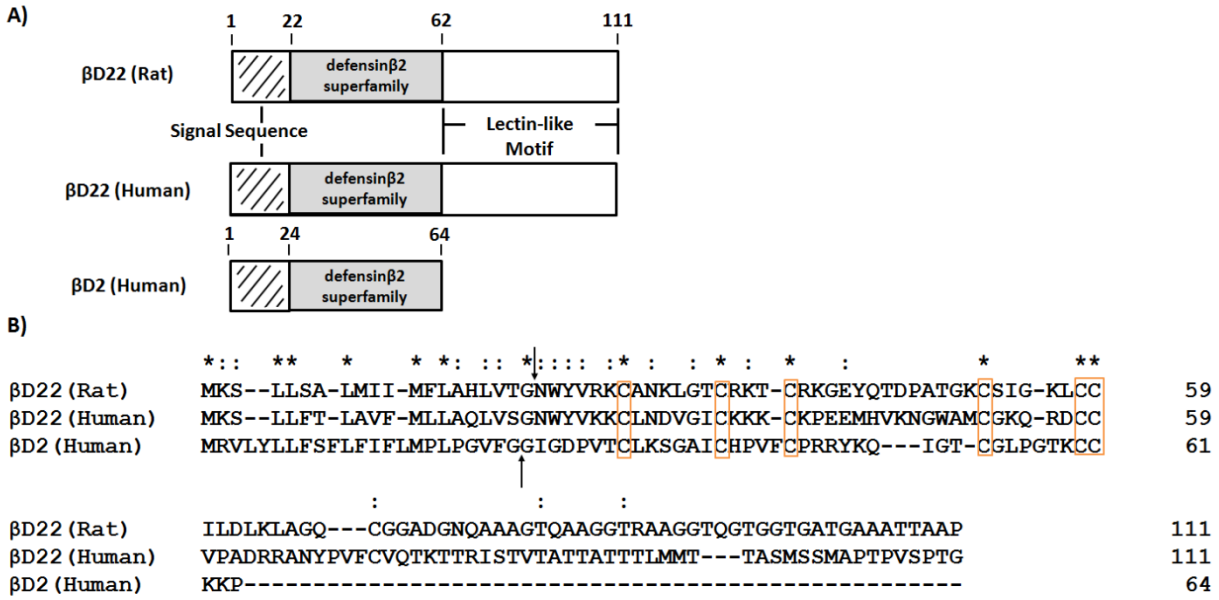

Figure 4. Model and sequence alignment of rat and human β-defensins.

A) Model comparison and B) sequence alignment of ratβD22, hβD22 and hβD2 peptides. In A, signal sequences are indicated by hatched boxes and defensin β2 superfamily motifs by gray boxes. In B, conserved residues across all three peptides are marked above with asterisks, and conserved β-defensin cysteine residues are boxed. Conserved residues between rat and human βD22 are marked above with colons. Arrows point to signal sequence cleavage sites.

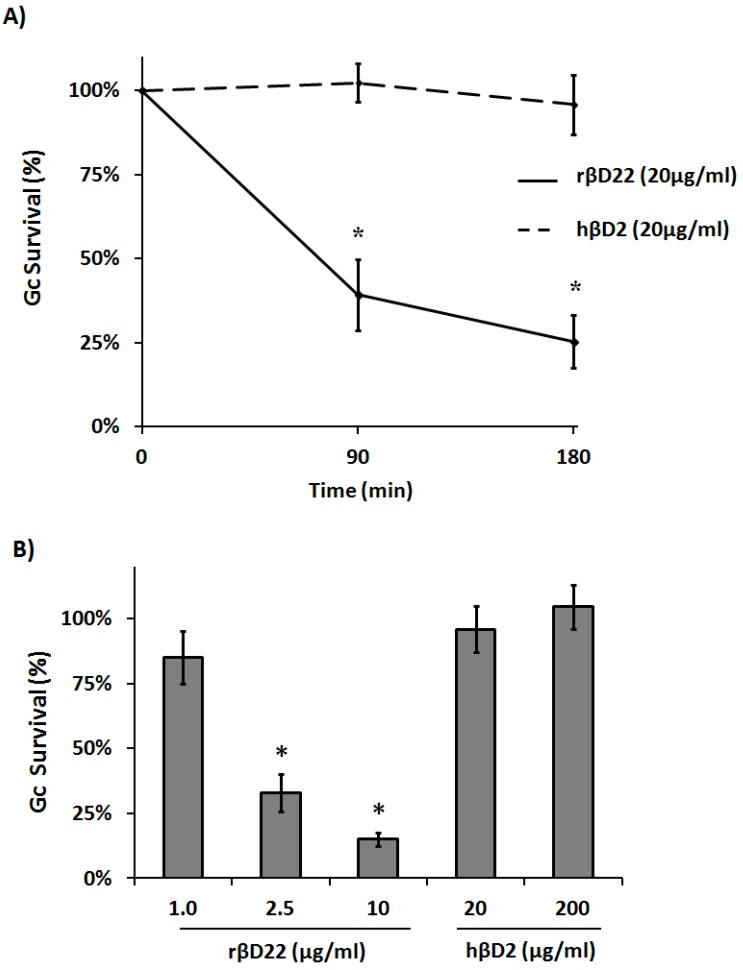

Figure 5. rβD22, but not hβD2, has concentration-dependent and time-dependent bactericidal activity against *N. gonorrhoeae.*

A-B) Gc was incubated with indicated concentrations of rβD22 or hβD2 for 1.5hr (A) or 3hrs (A and B). Survival at each time point and concentration is expressed relative to bacterial survival in the untreated control. A, *, $P < 0.05$ for rβD22 vs hβD2 at matched time points; Students two-tailed *t*-test, n=3. B, *, $P < 0.05$ for rβD22 at 2.5 or 10 μg/ml compared with rβD22 at 1 μg/ml and with hβD2 at 20 or 200 μg/ml; one-way ANOVA, n=3.

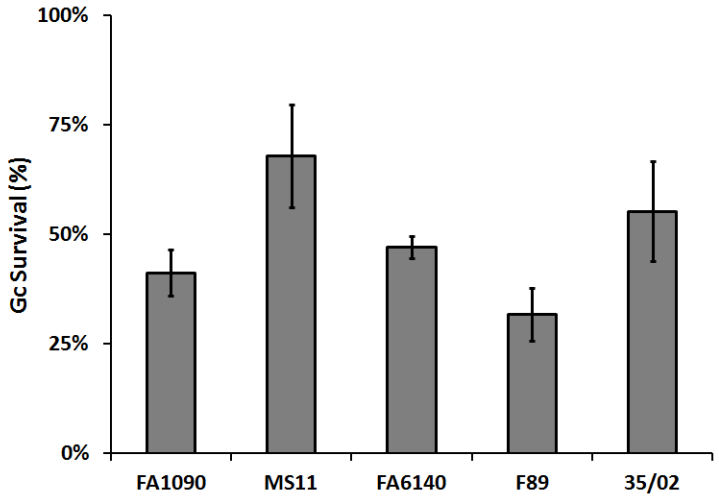

Figure 6. *N. gonorrhoeae* laboratory and multidrug-resistant strains are sensitive to killing by β-defensin 22.

The indicated strains of Gc were incubated with rβD22 (20μg/ml) for 3hrs. Survival of each strain is expressed relative to bacterial survival of the same strain in the untreated control. n=4.

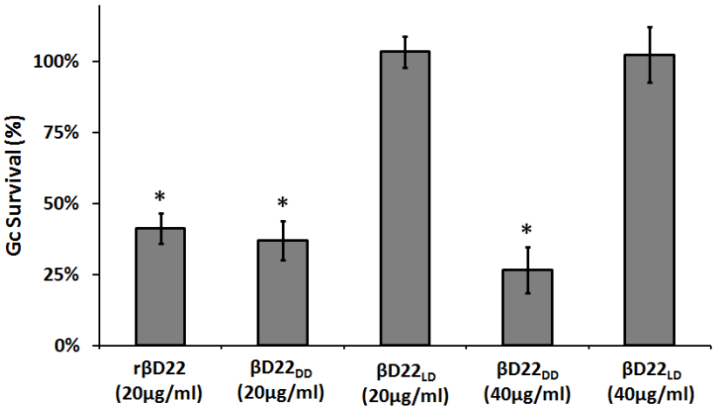

Figure 7. The βD22 defensin domain, but not the lectin domain, is important for antigonococcal activity.

Gc was incubated with full-length βD22, the N-terminal defensin domain (aa 22-61) or the C-terminal lectin domain (aa 62-111) for 3hrs. Survival is expressed relative to bacterial survival in the untreated control.*, $P < 0.05$ for rβD22 and $\beta D22_{DD}$ (all concentrations) compared with $\beta D22_{LD}$ (all concentrations); one-way ANOVA, n=5.

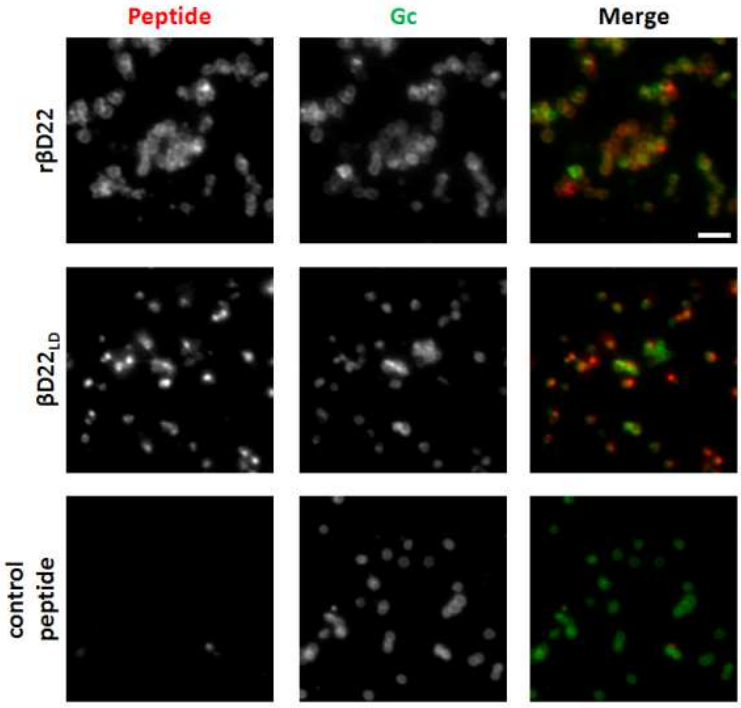

Figure 8. The lectin domain of βD22 mediates binding to intact Gc.

Gc was incubated with full-length βD22, the βD22 lectin domain, or a control peptide for 1 hour. All proteins were His-tagged. Bacteria were fixed with paraformaldehyde and incubated with anti-His-tag and anti-Gc antibodies, followed by fluorescently-tagged secondary antibodies. Gc appears green, and His-tagged proteins appear red. Scale bar = 2.5µm.

3. Chapter 3: The lipooligosaccharide-modifying enzyme LptA enhances gonococcal defense against human neutrophils

This chapter is modified from the article entitled, "the lipooligosaccharide-modifying enzyme LptA enhances gonococcal defence against human neutrophils." *Cell Microbiol.* 2015 Jun;17(6):910-21. doi: 10.1111/cmi.12411.

3.1 Introduction

Gonorrhea continues to be a global health concern. Over 106 million cases of gonorrhea are estimated annually worldwide, up from 88 million in 2011 (World Health Organization, 2011; World Health Organization, 2012). *Neisseria gonorrhoeae* (gonococcus, Gc) is the sole, causative agent of gonorrhea, and is one of three bacterial pathogens currently regarded as an "urgent," highest-level threat by the US Centers for Disease Control and Prevention (CDC, 2013). Gc has attained "superbug" status based on emerging resistance to third-generation cephalosporins – the last recommended line of disease treatment – and on the continued unavailability of a vaccine (CDC, 2011).

Symptomatic gonorrhea is characterized by a substantial influx of polymorphonuclear leukocytes (PMNs; neutrophils) to the site of infection. Gc can be recovered from PMN-rich gonorrheal exudates and primary human PMNs infected *ex vivo* (Criss and Seifert, 2012), but the defense mechanisms used by Gc to survive the diverse antimicrobial activities of PMNs are just starting to be defined. Lipooligosaccharide (LOS) is one of the few known virulence factors for Gc and the most abundant surface component (Hobbs *et al.*, 2013).*Neisserial* LOS is composed of lipid A that anchors the structure to the membrane, which is connected to the inner core sugars, two 3-deoxy-D-manno-octulosonic acid (KDO) residues and two heptoses, from which the outer-core oligosaccharide extends. LOS variation is an important determinant for Gc interactions with the host (Gotschlich, 1994; Shafer *et al.*, 2002; Tzeng

et al., 2005; Lewis *et al.*, 2009; Balthazar *et al.*, 2011). Many of the LOS outer-core biosynthetic genes are phase variable, and components of LOS can be enzymatically modified (Gotschlich, 1994; Shell *et al.*, 2002; Cox *et al.*, 2003). One of these modifications is the addition of phosphoethanolamine (PEA) to the 4' phosphate on lipid A, catalyzed by the phase-variable LOS phosphoethanolamine transferase A (LptA) (Cox *et al.*, 2003; Lewis *et al.*, 2009; Zughaier *et al.*, 2014).*lptA* mutant Gc is more susceptible to bacteriolysis by human complement, and both Gc and *Neisseria meningitidis* lacking *lptA* are more susceptible to killing by cationic antimicrobial proteins (CAMPs) (Tzeng *et al.*, 2005; Lewis *et al.*, 2009; Lewis *et al.*, 2013). *lptA* mutant Gc is attenuated in experimental male infection and cervicovaginal murine challenge (Hobbs *et al.*, 2013; Packiam *et al.*, 2014). *Neisseria* lipid A lacking the 4' PEA modification is also less immunostimulatory, causing lower levels of TNFα to be released by human monocytes and less induction of NFκB via Toll-like receptor 4 (TLR4) (John, Liu, and GA Jarvis, 2009; John, Liu, and Gary A Jarvis, 2009; Liu *et al.*, 2010; Packiam *et al.*, 2014). Most *Neisseria* commensal strains, with the exception of *N. lactamica*, do not encode a functional *lptA* gene, and this is hypothesized to contribute to their commensalism (John *et al.*, 2012).

Here, we investigated the contribution of LptA to Gc defense against killing by primary human PMNs. Expression of LptA enhanced Gc survival from the intracellular and extracellular antimicrobial activities of PMNs. Three mechanisms contributed to the survival advantage of LptA-expressing Gc after exposure to PMNs: increased resistance to PMN CAMPs, including CAP37/azurocidin, human neutrophil peptide-1 (HNP-1), and the serine protease cathepsin G; reduced residence in mature phagolysosomes; and increased resistance to the antimicrobial effects of neutrophil extracellular traps (NETs). These results highlight the importance of LptA for Gc survival during interaction with human PMNs.

3.2 Materials and Methods

3.2.1 *Bacterial strains and growth conditions*

Constitutively piliated, opacity protein-deficient ("Opaless") Gc of strain FA1090 served as the parent for these studies unless otherwise indicated (Ball and Criss, 2013). The phase-variable *lptA* gene (7-thymidine repeat) is in-frame in Opaless Gc. The Opaless Gc derivative constitutively expressing OpaD was previously described (Opaless::$opaD^{+}_{nv}$, (Ball and Criss, 2013). Gc was maintained on gonococcal medium base (BD Difco) with Kellogg's supplement I+II (GCB) (Kellogg *et al.*, 1963) and regularly grown in rich liquid medium (GCBL) for 16-20 h at 37°C/5%CO_2 (Criss and Seifert, 2008). FA19 parent and *ΔlptA* Gc were a kind gift of William Shafer (Emory University). TOP10 *E. coli* and *Staphylococcus aureus* ATCC 25923 were regularly cultured on Luria-Bertani (LB) agar and grown in LB broth for 16-20 h at 37°C. In these studies, Gc was not opsonized with human serum because of the reported high sensitivity of the *lptA* mutant to killing by human complement (Lewis *et al.*, 2013). *S. aureus* was opsonized with 20% freshly isolated human serum for 20 min at 37°C prior to experimental use.

3.2.2 *Construction of lptA mutant and complement strains*

FA1090 Gc was transformed with a plasmid containing insertionally inactivated *lptA*. The inactivated *lptA*gene fragment was constructed using overlap extension PCR (Heckman and Pease, 2007). A fragment of <1 kb comprised of the region upstream of *lptA* and the 5' end of the gene (F1) was amplified from Opaless Gc gDNA using the primer pair LPTAF (5'-GTT GCA GAC CGG TTC GAA TTT TGC-3') and LPTAF1R (5'-GCT TCT GTA TGG AAC GGG CAG TTA ACG ATG GGT TAC TGA TTT ATT GTT GCG G-3'). A second fragment, containing the 3' end of *lptA* and downstream sequence (F2), was amplified using the primer pair LPTAF2F (5'-GCT CAC AGC CAA ACT ATC AGG TAG CGC TCT CAACCT GCC CGA ATA CTG C-3') and LPTAR (5'-TTC AAC ACA TCG CGA AAA CGT TGC-3'). An omega-spectinomycin resistance cassette (Ω) was amplified using the primer pair ΩLPTAF(5'-CCG CAA CAA TAA ATC AGT AAC CCA TCG TTA ACT GCC

CGT TCC ATA CAG AAG C-3') and ΩLPTAR (5'-GCA GTA TTC GGG CAG GTT GAG AGC GCT ACC TGA TAG TTT GGC TGT GAG C-3'). F1 and Ω PCR fragments were mixed in equimolar ratios and amplified using LPTAF and ΩLPTAR. The overlap extension PCR product (F1- Ω) and F2 were ligated separately into pCR™4Blunt-TOPO® vector (Life Technologies), following manufacturer's suggestions. TOP10 *E. coli* were transformed with the F1- Ω ligation product or with the F2 ligation product and selected for on LB agar containing 100 μg ml^{-1}spectinomycin or 60 μg ml^{-1} kanamycin, respectively. Transformed colonies were grown in LB broth, and the plasmids were purified using the QIAPrep Spin Miniprep Kit (Qiagen). F1-Ω-pBLUNT was restriction enzyme-digested using AfeI and XbaI (New England Biolabs) and F2 was restriction enzyme-digested using SpeI and AfeI (New England Biolabs) The F1-Ω insert and F2-pBLUNT were ligated together with T4 DNA Ligase (New England Biolabs), following manufacturer's suggestions. F1-Ω-F2 (*lptA::spc*)-pBLUNT was purified from TOP10 *E. coli* that had been transformed with the ligation product and selected for on 100 μg ml^{-1} spectinomycin. The plasmid construct was introduced into Opaless and Opaless::$opaD^{+}_{nv}$ Gc by natural transformation on solid medium (Stohl and Seifert, 2001), and transformed bacteria were selected on GCB agar containing 30 μg ml^{-1} spectinomycin. Transformants were confirmed by PCR and sequencing with the primer pair LPTASEQ2F (5'-GTG CGG CGG TGT CTT ACC AAG-3') and LPTASEQ3R (5'-CGA TTT CGT TGG TAT CGC ATG TC-3').

To complement FA1090*ΔlptA::spc,* the bacteria were transformed with the pGCC4 complementation plasmid containing the isopropyl-β-D-galactosidase (IPTG)-inducible *lptA* gene (from William Shafer, Emory University; as described in (Lewis *et al.*, 2009)). Transformants were selected on GCB agar containing 0.25 μg ml^{-1} erythromycin. The *lptA* complement was induced by growing the bacteria in rich liquid medium containing 250 μM IPTG for 2.5 h. At this concentration, the induced bacteria showed similar resistance to polymyxin B as the isogenic parent (Figure 17).

3.2.3 *PMN Isolation*

Venous blood was drawn from healthy human donors that had given informed consent in accordance with a protocol approved by the Virginia Institutional Review Board for Health Sciences Research. Heparinized blood, depleted of erythrocytes by dextran sedimentation, was then purified over a Ficoll-Hypaque gradient to isolate PMNs (Stohl *et al.*, 2005). PMNs were resuspended in Dulbecco's PBS (without Calcium and Magnesium; Thermo Scientific) containing 0.1% dextrose, and kept on ice for < 2 h before use. Preparations were normally >95% PMNs by phase-contrast microscopy.

3.2.4 *PMN Antimicrobial Assay*

IL-8-treated (10nM, R&D Systems) PMNs (10^6 per coverslip) were allowed to adhere to tissue culture treated 13mm plastic coverslips in RPMI (Mediatech) containing 10% fetal bovine serum (FBS; Thermo Scientific), for 1 h at 37°C/5% CO_2. PMNs were then synchronously infected with exponential-phase Gc at a multiplicity of infection of 1-5 as described previously (Criss *et al.*, 2009). At indicated time points, PMNs were lysed in 1% saponin, and Gc was plated on GCB agar. Percent survival was calculated as the CFU at each time divided by the CFU at time 0.

3.2.5 *PMN granule extract*

Proteins were extracted from PMN cytoplasmic granules using a protocol adapted from Rest (Rest, 1979). Freshly isolated PMNs were resuspended in 0.34 M sucrose and sheared by 15-25 passages through a ball bearing homogenizer until 90-95% breakage was achieved, as indicated by trypan blue staining. The homogenate was centrifuged at 200 x *g* for 15 min to remove nuclear and cellular debris, then at 20,000x*g*for another 15 min to pellet the granules. Pellets were resuspended in acetate buffer (0.2M, pH 4.0) and acid-extracted overnight at 4°C twice, then clarified by high-speed centrifugation as above. The extract was stored in acetate buffer at 4°C and was used within 4 weeks of purification. The

extract was dialyzed against PBS (8mM NaCl, 12mM K_2HPO_4, 4mM KH_2PO_4; pH 7.2) overnight in 3,500 MWCO dialysis tubing (Spectra/Por) prior to experiments.

3.2.6 *Viability of bacteria in association with PMNs*

Baclight viability dyes (Invitrogen) were used to assess Gc survival intracellularly and extracellularly in the presence of PMNs as described (Criss *et al.*, 2009). In some experiments, PMNs were treated with 1x Protease Inhibitor Cocktail Set V containing AEBSF, aprotinin, E-64, and leupeptin (Calbiochem) during attachment to coverslips and throughout the infection.

3.2.7 *CAMP Bactericidal Assay*

Mid-logarithmic Gc (5 x 10^5) was exposed to the indicated concentrations of Polymyxin B (Alexis Biomedicals), CAP37 (Sigma), HNP-1 (Sigma), Cathepsin G (MP Biomedicals), LL-37 (generously provided by William Shafer, Emory University) or PMN granule extract in 0.2x strength GCBL for 45 min at 37°C/5%CO_2. Cathepsin G was also incubated with 1x protease inhibitors for 30 min prior to and throughout the assay. Survival was calculated by enumerating CFU at time 0 and time 45 min to yield percent Gc survival for each concentration, then normalized to Gc survival in the untreated control (set to 100%).

3.2.8 *PMN Phagosome Maturation*

Immunofluorescence imaging of granule enrichment at Gc phagosomes was performed as described (Johnson and Criss, 2013). Prior to infection, Gc were incubated with CFSE to stain total bacteria. Extracellular Gc were detected using an anti-Gc antibody (Biosource) and secondary Alexa Fluor-coupled secondary antibody without PMN permeabilization. Granule enrichment was assessed in permeabilized PMNs with a monoclonal antibody against neutrophil elastase for primary granules (AHN10, Invitrogen) or a polyclonal anti-lactoferrin antibody (MP Biomedicals) for secondary granules,

followed by Alexa Fluor-coupled secondary antibody. Cells were examined on a LSM510 confocal laser scanning microscope (Zeiss). Bacterial phagosomes were scored as positive for granule enrichment when the antibody staining for the granule component surrounded ≥50% of the bacterial circumference.

3.2.9 *PMN supernatant antimicrobial assay*

10^6 PMA-primed (10nM, Sigma) PMNs were allowed to adhere to tissue culture-treated 13mm plastic coverslips in 0.4 ml RPMI (Mediatech) containing 10% FBS for 30 min at 37°C/5% CO_2. The supernatant was filtered (0.2 μM) to remove any PMNs. Gc (10^6) was incubated with 0.1 ml supernatant or RPMI for 45 min at 37°C/5%CO_2. Bacteria were plated on GCB agar, and incubated overnight. Survival was calculated by dividing the percent survival of CFU in a treated condition by the percent survival of CFU in the untreated control**.**

3.2.10 *Gc survival in NETs*

Adherent PMNs were exposed to 10nM PMA for 30 min, then treated with cytochalasin D (10 μg ml^{-1}, Sigma) in the presence or absence of DNaseI (1 U ml^{-1}, NEB) for 20 min at 37°C/5%CO_2 or were treated with DNAse I (1 U ml^{-1}) after the 1 h infection. PMNs were then exposed to Gc at MOI = 1 for 1 h. Bacterial survival was calculated relative to CFU enumerated at 0 min for each condition.

3.2.11 *ROS detection*

PMNs were left untreated or exposed to Gc or serum-opsonized *S. aureus* at an MOI of 100. ROS production was measured by luminol-dependent chemiluminescence as described previously (Ball and Criss, 2013).

3.2.12 *Bacterial growth*

Liquid-grown Gc was diluted to an O.D. of 0.07 in modified GCBL. Liquid cultures were grown, shaking, for 4 h at 37 °C. Growth was measured hourly by optical density and plating of bacteria on GCB agar for CFU enumeration.

3.2.13 *Statistics*

Values are mean ± standard error of three independent replicates (unless otherwise noted). Significance was assessed using the student's two-tailed *t*-test. A P-value of less than 0.05 was considered significant.

3.3 Results

3.3.1 *LptA improves survival of N. gonorrhoeae exposed to human PMNs*

Piliated, opacity protein-deficient Gc of strain FA1090, an isogenic *lptA* mutant (*ΔlptA*), and *ΔlptA* complemented with full-length *lptA* under the control of an IPTG-inducible promoter (*lptA*$^{+}$) were assessed for their survival in the presence of adherent, IL-8-treated human PMNs (Figure 9). *lptA*$^{+}$ Gc was induced with 250 µM IPTG, which conferred similar resistance to the model cationic antimicrobial peptide polymyxin B as exhibited by parent bacteria (Figure 17). After exposure to PMNs, approximately 60% of the parent and *lptA*$^{+}$inocula were recovered 30 min after exposure and increased thereafter, reaching approximately 150% of the inocula by 2 h. *ΔlptA* Gc exhibited a similar decline in survival over 30 min, but unlike the parent and *lptA+*strains, it failed to recover at later times, with statistically significant decreases in survival measured at 60 and 120 min post-infection. *ΔlptA* bacteria remained viable in infection media without PMNs over the same time period (data not shown) and did not show any significant differences in growth in rich liquid media compared to parent and *lptA*$^{+}$ Gc (Figure 18).

3.3.2 *LptA is important for survival of Gc exposed to primary human PMNs*

PMNs are equipped with a variety of killing mechanisms that are released both intra- and extracellularly. To further characterize the *ΔlptA* survival defect, we used dyes that differentially report on the viability of individual bacteria, in combination with a fluorescently-labeled lectin, to differentiate live from dead and intracellular from extracellular Gc (Figure 10A). *ΔlptA* Gc exhibited decreased survival both intracellularly and extracellularly compared to parent and *lptA*$^{+}$ bacteria after 1 h infection (Figure 10B). There was no significant difference in internalization by PMNs among the three strains (Figure 10C). These results indicate that Gc expressing LptA is protected against the killing mechanisms of PMNs in both intracellular and extracellular environments.

3.3.3 *LptA protects Gc from the bactericidal activity of non-oxidative components of human PMNs*

PMNs use non-oxidative components including proteases and antimicrobial peptides to combat Gc (Casey *et al.*, 1986; W.M. Shafer, Martin, *et al.*, 1986; Criss *et al.*, 2009; Johnson and Criss, 2013). It has been shown that *lptA* mutants in Gc and *N. meningitidis* are more sensitive to some of these components, due to loss of PEA on lipid A (Tzeng *et al.*, 2005; Lewis *et al.*, 2009). To test the hypothesis that the reduced survival of *ΔlptA* Gc after exposure to PMNs was due to increased sensitivity to PMN granule proteins, parent, *ΔlptA,* and *lptA*$^{+}$ Gc were exposed to an extract made from the cytoplasmic granules of PMNs. The purified PMN extract exhibited anti-gonococcal activity, and *ΔlptA* Gc was significantly more susceptible to the extract than parent Gc (Figure 11A). We therefore examined components found in PMN granules for their activity against parent and *ΔlptA* Gc. The *lptA* mutant had increased sensitivity to all three of the primary (azurophilic) granule components tested (Figure 11B-D). CAP37 had modest antimicrobial activity against parental Gc, in keeping with previous reports (W M Shafer *et al.*, 1986), but was significantly more active against Δ*lptA* Gc (Figure 11B). The α-defensin HNP-1 had no activity against parental Gc, as previously reported (Qu *et al.*, 1996); however, *ΔlptA* Gc was

sensitive to it (Figure 11C). The serine protease cathepsin G had minor bactericidal activity against parent Gc, but was significantly more effective at killing *ΔlptA* bacteria (Figure 11D). The increased sensitivity of *ΔlptA* Gc to cathepsin G was due in part to its protease activity (Figure 11D). In contrast, the contribution of LptA to protection from the CAMP LL-37 depended on the bacterial strain background: there was no difference in sensitivity to LL-37 for FA1090 parent and *ΔlptA* Gc (Figure 11E), but as previously reported (Lewis *et al.*, 2009; Lewis *et al.*, 2013), an *lptA* mutant in strain FA19 was significantly more sensitive to LL-37 compared with its parent (Figure 11F). FA1090 *ΔlptA* Gc was also significantly more sensitive to polymyxin B than its parent (Figure 17). Taken together, these results show that expression of LptA increases Gc defense against some of the non-oxidative components made by PMNs, and this is influenced by the bacterial genetic background.

3.3.4 *LptA is important for Gc defense against non-oxidative components produced by PMNs*

We previously showed that primary granule serine protease activity contributes to the killing of Gc inside PMNs (Johnson and Criss, 2013). To test whether serine proteases contribute to the increased sensitivity of *ΔlptA* Gc to PMNs, bacterial survival was measured in PMNs treated with a protease inhibitor cocktail. Treatment with the protease inhibitors significantly improved the intracellular and extracellular survival of *ΔlptA* Gc after exposure to PMNs, to levels indistinguishable from parent Gc (Figure 12). Thus serine proteases such as cathepsin G also have antigonococcal activity in the context of PMN infection, and LptA is important for Gc defense against them.

We also explored whether there were differences in the ability of PMNs to mount an oxidative burst after exposure to *ΔlptA* Gc. As we previously reported, the opacity protein-deficient parent Gc strain used here did not stimulate release of ROS by PMNs (Figure 13). Neither Δ*lptA* nor *lptA*$^{+}$ Gc induced detectable ROS from PMNs, but PMNs were otherwise able to generate ROS, shown

using *Staphylococcus aureus* as a positive control (Figure 13). Thus differences in ROS production do not contribute to the survival defect of *ΔlptA* Gc after exposure to PMNs.

3.3.5 *LptA-deficient N. gonorrhoeae are more frequently found in mature, primary granule-positive PMN phagolysosomes*

PMN phagosomes containing opacity protein-negative Gc exhibit delayed maturation due to reduced fusion with primary granules, but not secondary (specific) granules (Johnson and Criss, 2013). Phagosomes containing *ΔlptA* Gc exhibited a statistically significant increase in primary granule enrichment compared to the parent, with no appreciable difference in secondary granule enrichment (Figure 14A-D).

To test if increasing the exposure of *ΔlptA* Gc to primary granule components would further compromise its survival, the *lptA* deletion was introduced into the $opaD^{+}_{nv}$ Gc background. We recently reported that PMN phagosomes containing Opa^{+} Gc have significantly increased enrichment of primary granule components (Johnson *et al.*, 2015). The survival of $opaD^{+}_{nv}$ Δ*lptA* Gc was reduced relative to Δ*lptA* Gc and $opaD^{+}_{nv}$ Gc after exposure to PMNs, although the differences were not statistically significant (Figure 14E). Taken together, we conclude that increased residence of *ΔlptA* Gc in primary granule-rich phagolysosomes, in addition to the increased sensitivity of *ΔlptA* Gc to primary granule components, contributes to the survival defect of the mutant inside PMNs.

3.3.6 *LptA protects Gc from killing by extracellular PMN components*

Since *ΔlptA* Gc is also more sensitive to extracellular killing by PMNs (Figure 10), we tested the susceptibility of parent and *ΔlptA* Gc to the two approaches used by PMNs to combat extracellular microorganisms: degranulation and NET formation (Johnson and Criss, 2011). First, PMNs were stimulated with PMA to promote exocytosis of all granule types, and Gc were incubated with the

degranulated supernatant. *ΔlptA* Gc was significantly more sensitive to the degranulated supernatant than the parent, which had no loss in viability (Figure 15). Second, to induce NETs, PMNs were stimulated with PMA, in the presence of cytochalasin D to block phagocytosis. *ΔlptA* Gc was more sensitive than parent Gc to killing by PMNs that release NETs. Importantly, *ΔlptA* Gc survival was rescued by the addition of DNAse I to degrade NET DNA (Figure 16). Treatment of NETs with DNAse I after infection did not rescue the survival defect of Δ*lptA* Gc, implying that NETs had antimicrobial activity against *ΔlptA* Gc, rather than trapping the mutant and preventing accurate colony counts (Figure 16). Taken together, these results show that LptA protects Gc from degranulation and NETs, thereby hampering the ability of PMNs to combat extracellular Gc.

3.4 Discussion

The mechanisms Gc uses to resist the robust antimicrobial activities of PMNs are beginning to be elucidated. The LOS-modifying enzyme LptA is only one of a few known virulence factors that promote Gc pathogenesis, since LptA is crucial for Gc survival following infection of the human male urethra or the murine cervix (Hobbs *et al.*, 2013; Packiam *et al.*, 2014). Here we demonstrate that LptA also improves Gc defense from killing by primary human PMNs, and does so in three distinct ways: increasing Gc resistance to non-oxidative PMN components, including from degranulated PMNs, avoiding Gc residence in mature phagolysosomes, and enhancing Gc survival in NETs. Gc with PEA-modified lipid A also shows increased binding of factor H and C4BP (Lewis *et al.*, 2013), implying that LptA would also contribute to the resistance of Gc to complement-mediated killing and opsonophagocytosis by PMNs. Through these complementary mechanisms, LptA provides a crucial advantage for survival of Gc after exposure to PMNs, thus facilitating bacterial persistence in its obligate human hosts.

Given that LptA has been reported to provide Gc with defense against CAMPs *in vitro* (Tzeng *et al.*, 2005; Balthazar *et al.*, 2011; Packiam *et al.*, 2014), we hypothesized that LptA would help protect Gc

from PMNs by enhancing its resistance to PMN-derived CAMPs. We found that LptA-expressing Gc is significantly more resistant to killing by a purified PMN granule extract as well as the degranulated supernatant from activated PMNs. PMN granules contain a variety of membrane-associated and soluble components designed to detect and eliminate threats to the host. Prevalent among these are CAMPs such as α-defensins, CAP37, and serine proteases. CAMPs disrupt the bacterial membrane through charge-charge interactions and also have regulatory effects on immune events (Steinstraesser *et al.*, 2011). Here we found that LptA protects Gc from killing by two CAMPs, CAP37 and HNP-1. Although CAP37 was one of the first antigonococcal proteins to be identified (Shafer *et al.*, 1984), LptA is the first defined Gc gene product to be shown to modulate Gc sensitivity to CAP37. Gc is highly resistant to HNP-1 (Qu *et al.*, 1996), and here we show that one of the major contributors to this resistance is LptA. Serine proteases can have direct antimicrobial activity, process precursor proteins into their active forms (Sørensen *et al.*, 2001) and facilitate innate and adaptive immune responses (Pham, 2006; Mantovani *et al.*, 2011). We found that LptA expression was particularly important for Gc defense against killing by cathepsin G, due in part to its serine protease activity. Since the protease inhibitor cocktail used here can inhibit all PMN serine proteases, LptA may also help defend Gc against neutrophil elastase and proteinase 3, although they have not been reported to have direct antigonococcal activity akin to cathepsin G.

The role of serine proteases in the killing of *ΔlptA* Gc may be linked not only to their direct antimicrobial activity, but also to their processing of host precursor proteins into their active antimicrobial forms. Cathepsin G has protease-independent antigonococcal activity (W.M. Shafer, Onunka, *et al.*, 1986) by its similarity in sequence and predicted structure with other PMN CAMPs (Shafer *et al.*, 1993). Serine protease activity may be needed to cleave full-length cathepsin G into a bioactive CAMP-like fragment that is more active against *ΔlptA* bacteria. Gc is also sensitive to the CAMP LL-37, which is cleaved from the hCAP18 precursor by the serine protease proteinase 3 (Sørensen *et al.*,

2001). Strikingly, we found that loss of *lptA* had no effect on the sensitivity of Gc strain FA1090 to killing by LL-37. This is in contrast to increased sensitivity to LL-37 by *ΔlptA* Gc in the FA19 strain background (Packiam *et al.*, 2014). This discrepancy may be due to differences in expression of the MtrCDE multidrug efflux pump in these two strain backgrounds. FA1090 has a natural mutation in the *mtrA* activator of MtrCDE expression, rendering the strain more sensitive to LL-37 and other compounds that are normally expelled by the pump (Rouquette *et al.*, 1999). Thus FA1090 may already have such sensitivity to LL-37 that loss of *lptA* has no increased effect. FA1090 and FA19 also produce different oligosaccharide α chains on their LOS species, which may affect intrinsic sensitivity of these strains to LL-37 and other CAMPs (Veal *et al.*, 2002; Hobbs *et al.*, 2013).

Since our working hypothesis was that LptA protects Gc from PMNs by providing defense against PMN-derived CAMPs, we were surprised to find that LptA also contributed to Gc residing in immature phagosomes inside PMNs. We previously reported that the fraction of Gc surviving inside PMNs are found in phagosomes that exhibit delayed fusion with primary granules to avoid becoming degradative phagolysosomes (Johnson and Criss, 2013). Residence in immature phagosomes did not require actively growing Gc, suggesting a surface structure influenced phagosome composition, perhaps by affecting the bacterial entry process. Our findings suggest that LOS may be one such structure, and LOS composition, including 4' PEA decoration of lipid A, affects PMN processes leading to phagosome-granule fusion. There are at least two possibilities that could explain how LptA-modified LOS affects phagocytosis and phagosome dynamics. First, direct detection of LptA-modified LOS by PMNs could modulate signaling events that are important for phagosome-granule fusion. Second, LptA-modified lipid A could affect the surface presentation of LOS oligosaccharide chains or other outer membrane components, which are the structures that affect PMN activation and granule mobilization. In either scenario, since primary granules contain cathepsin G and other PMN serine proteases, we conclude that the decreased survival

of *ΔlptA* Gc inside PMNs is due to increased exposure to these components as well as increased intrinsic susceptibility to them.

LptA also contributes to Gc survival extracellularly after PMN challenge by protecting the bacteria from the antimicrobial components released by exocytosis and on NETs. Primary human PMNs release NETs after exposure to Gc (Juneau *et al.*, 2015). Loss of *lptA* in the related bacterium *N. meningitidis* has been shown to decrease bacterial growth in the presence of NETs, due to NET-associated CAMPs such as cathepsin G (Lappann *et al.*, 2013). We found that *ΔlptA* Gc has an extracellular survival defect in the presence of PMNs that had undergone NET release. Inhibiting the protease activity of cathepsin G and digesting the PMN NET backbone with DNase both rescue the survival of extracellular *ΔlptA* Gc. Taken together, we conclude that LptA expression improves extracellular survival of Gc against concentrated PMN CAMPs, including those incorporated into NETs.

The role of LptA in *Neisserial* biology is intriguing. Most commensal *Neisseria* strains do not encode a functional *lptA* gene (John *et al.*, 2012), while Gc and *N. meningitidis* do (Cox *et al.*, 2003; Tzeng *et al.*, 2005; Lewis *et al.*, 2009). 4' PEA-modified lipid A is a better ligand for TLR4 and thus initiates stronger inflammatory signals than unmodified *Neisserial* lipid A (John, Liu, and GA Jarvis, 2009; John, Liu, and Gary A Jarvis, 2009; Liu *et al.*, 2010; Packiam *et al.*, 2014). This would seem to be counterintuitive: while many bacterial pathogens attempt to escape detection by their hosts, the pathogenic *Neisseria* produce LOS species that are pro-inflammatory and likely to contribute to the influx of PMNs during acute gonorrhea or meningitis. We posit that LptA is an important virulence determinant for the pathogenic *Neisseria,* since the same LOS modification that promotes a highly inflammatory, PMN-rich environment confers resistance to the CAMPs and other antimicrobial products found within it. LptA-expressing Gc that are engulfed by PMNs avoid trafficking into mature PMN phagolysosomes and have increased resistance to the CAMPs encountered in phagosomes. Gc that

remain extracellular to PMNs can gather nutrients from surrounding host tissue that has been damaged or made leaky as a result of the potent PMN response, while resisting killing by NETs and exocytosed CAMPs (Criss and Seifert, 2012). Thus LptA expression is a double-edged sword during gonorrheal infection, contributing in multiple ways to the extraordinary success of Gc in the human population.

3.5 Figures

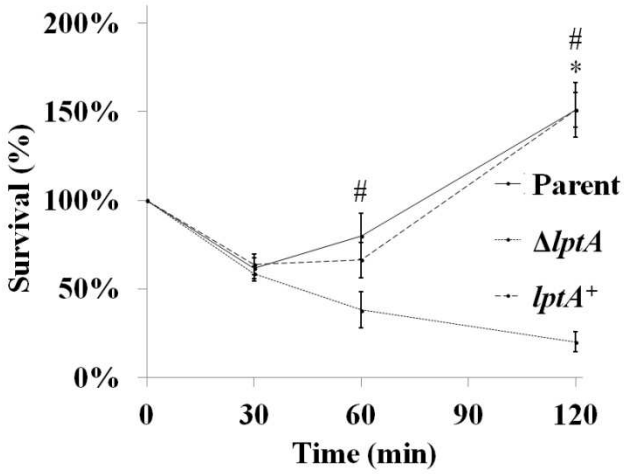

Figure 9. LptA is important for survival of Gc exposed to primary human PMNs.

Isogenic FA1090 parent, *lptA* mutant (*ΔlptA*), and *lptA* complement (*lptA+*) Gc were exposed to adherent, IL-8-primed PMNs. Bacterial survival was calculated by enumerating CFU in PMN lysates at 30, 60, and 120 min divided by CFU at 0 min. *, P<0.05 for parent *vs. ΔlptA*(*) and #, *P*< 0.05 for *lptA+ vs. ΔlptA*, two-tailed *t* test, n=4 experiments.

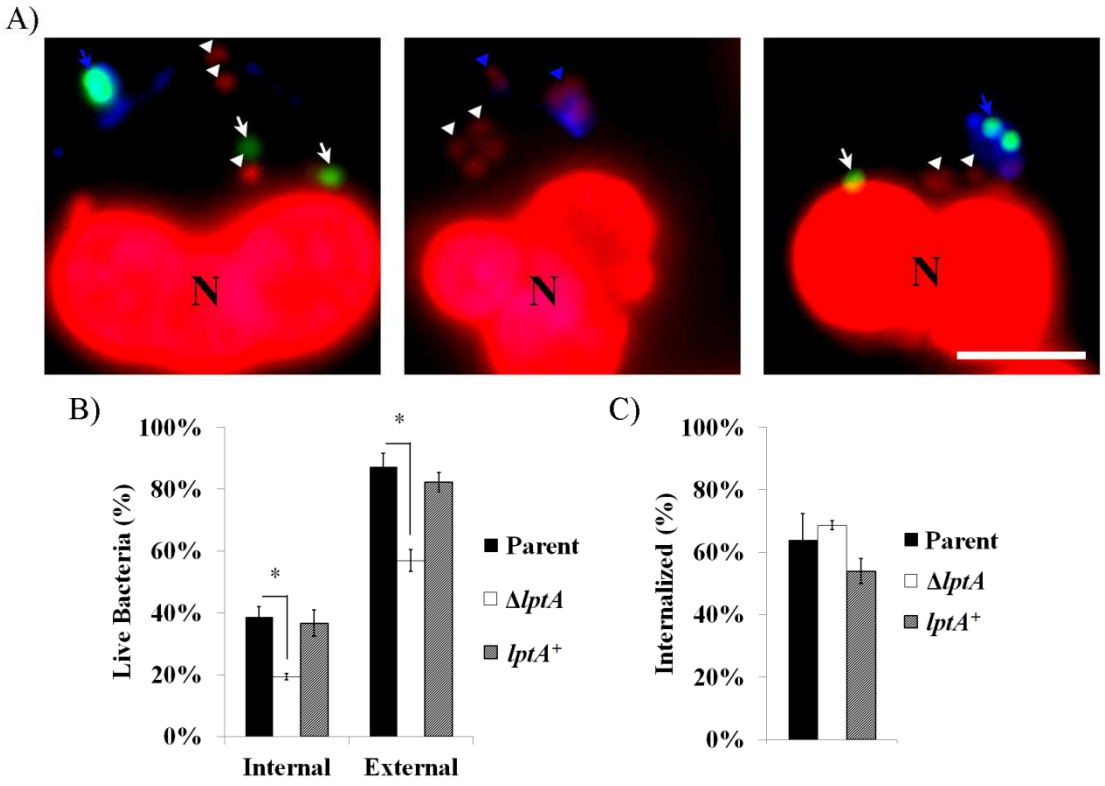

Figure 10. LptA improves survival of Gc both intracellularly and extracellularly when exposed to PMNs. Parent, *ΔlptA,* and *lptA+* Gc were exposed to adherent PMNs for 1 h. A) Gc was stained with an Alexa Fluor 647 conjugated soybean lectin (blue) to detect extracellular bacteria. PMNs were then permeabilized with saponin and exposed to Baclight viability dyes Syto9 (green) and propidium iodide (red) to discriminate viable from non-viable bacteria, respectively. White arrowheads indicate intracellular non-viable Gc, and white arrows indicate viable Gc. Blue arrowheads and arrows indicate extracellular non-viable and viable Gc, respectively. PMN nuclei were also PI+ (N). B) Bacterial viability was quantified for both intracellular and extracellular Gc and is expressed as the percent of total intracellular or extracellular Gc per strain. C) Percent bacterial internalization was calculated by dividing the number of intracellular bacteria (viable and non-viable) by the total number of cell-associated bacteria. Scale bar, 5µm. *, P<0.05 for parent *vs. ΔlptA*; two-tailed *t*-test, n= 3 experiments.

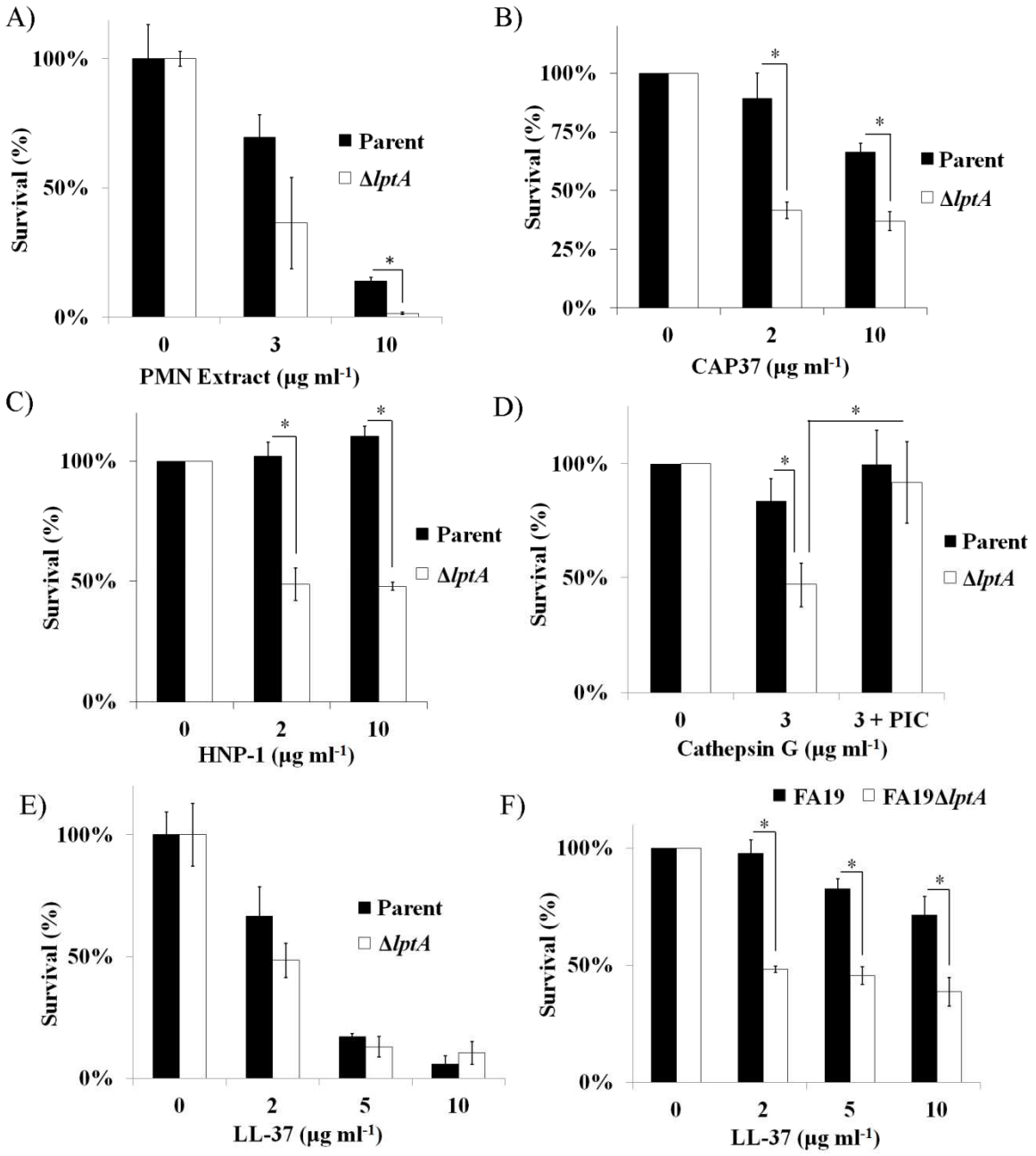

Figure 11. LptA is important for Gc defense against non-oxidative components produced by PMNs.

Parent and *ΔlptA* Gc in the FA1090 genetic background were exposed to the indicated concentrations of A) purified PMN extract, B) CAP37,C) HNP-1, D) Cathepsin G (+/− 1x protease inhibitor cocktail, PIC) or E) LL-37. In F) parent and *ΔlptA* Gc of strain FA19 were exposed to the indicated concentrations of LL-37. Survival at each concentration is expressed relative to bacterial survival in the untreated control. *, P<0.05 for indicated comparisons; Student's two-tailed *t* test, n=3 biological replicates per strain.

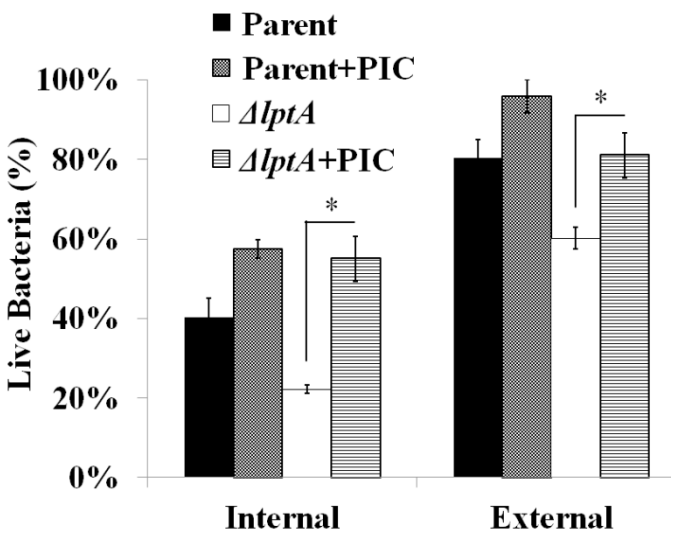

Figure 12. **LptA protects *N. gonorrhoeae* from killing mediated by protease activity in PMNs.**

Adherent PMNs were pre-treated with protease inhibitor cocktail (PIC) or mock-treated, then exposed to Gc for 1 h. Intracellular and extracellular Gc viability was measured using fluorescent dyes as in Figure 10. *, P<0.05 for *ΔlptA vs. ΔlptA* + PIC; two-tailed *t*-test, n=3 experiments.

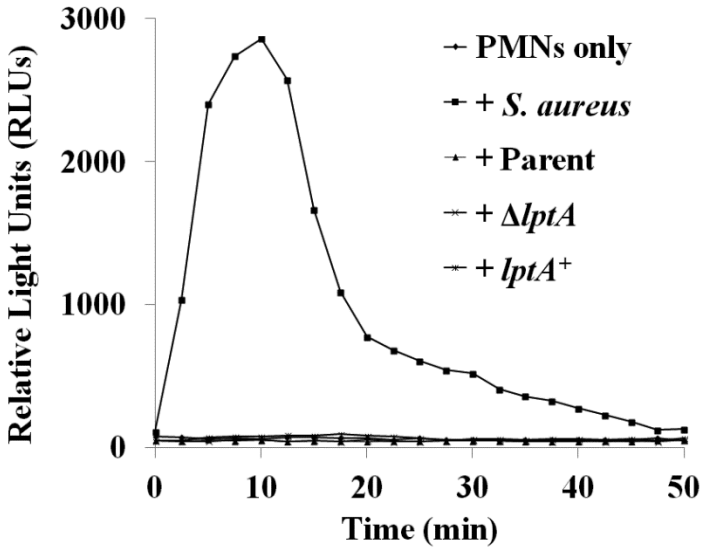

Figure 13. No PMN oxidative burst in response to parent or *ΔlptA* Gc.

PMNs were uninfected or exposed to the indicated Gc strains or opsonized S. aureus. The production of ROS was detected by luminol-dependent chemiluminescence over time. The graph is a representative replicate from one of three experiments.

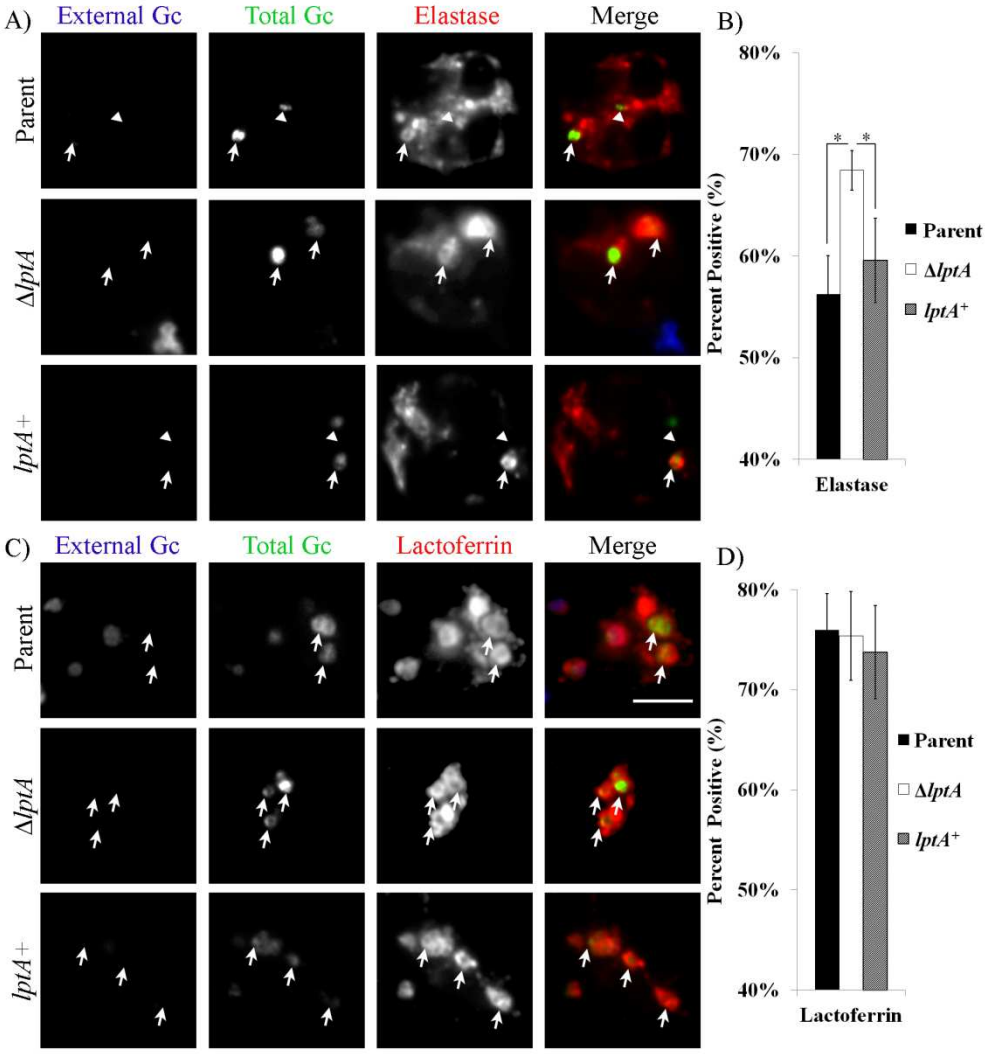

Figure 14. **Decreased maturation of phagosomes containing LptA-expressing Gc**.

CFSE-stained parent, *ΔlptA,* and *lptA+* Gc were exposed to adherent PMNs for 1 h. Extracellular and intracellular Gc were identified by accessibility to an anti-Gc antibody before and after PMN permeabilization. Granule enrichment was assessed as described in *Experimental Procedures* using antibodies against A) neutrophil elastase for primary granules or C) lactoferrin for secondary granules. Extracellular Gc appears blue and green, intracellular Gc appears green only, and granule proteins appear red. Granule enrichment was quantified for B) neutrophil elastase and D) lactoferrin. *, P < 0.05 for the indicated pairs by two-tailed *t*-test, n=3 experiments. E) Parent, Δ*lptA*, $opaD^{+}_{nv}$ and $opaD^{+}_{nv}$ Δ*lptA* Gc were exposed to PMNs, and Gc survival over time was enumerated as in Figure 9.*, *P* < 0.05 for parent *vs* . Δ*lptA*, $opaD^{+}_{nv}$ or $opaD^{+}_{nv}$ Δ*lptA*); Student's two-tailed *t*-test, n=3 experiments.

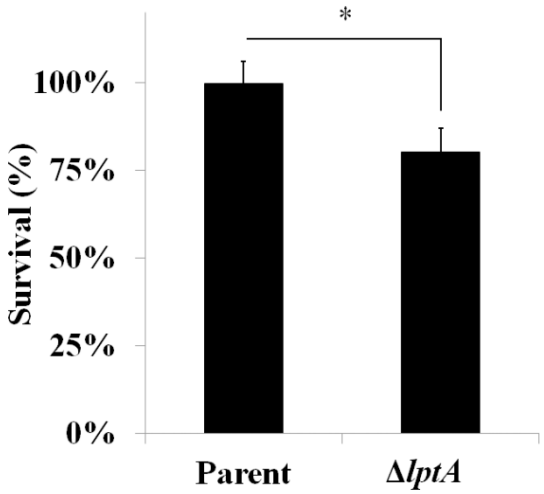

Figure 15. LptA improves resistance of Gc to killing by the degranulated supernatant of human PMNs.
Parent and *ΔlptA* Gc were exposed to supernatant from PMA-treated PMNs for 45 min (equivalent of supernatant from 2.5 x 10^5 degranulated cells). The percent of bacteria surviving supernatant treatment is expressed relative to the no treatment control. *, $P < 0.05$; Student's two-tailed *t*-test, n=3 experiments.

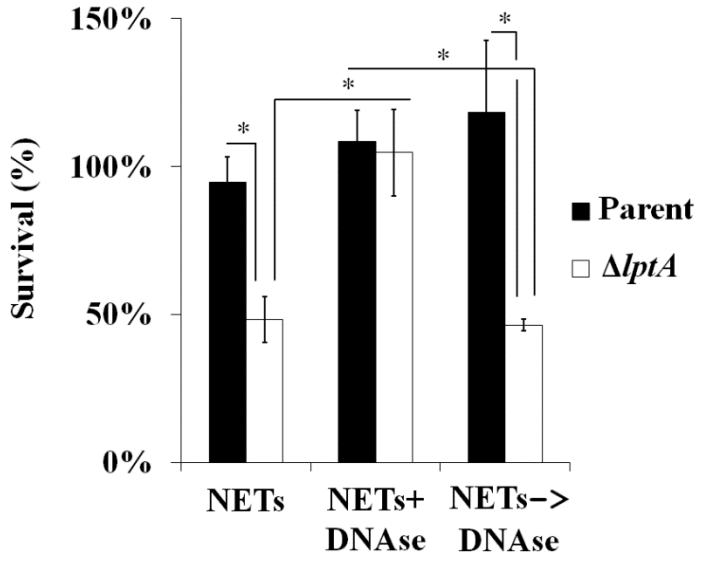

Figure 16. LptA enhances the resistance of Gc to the antimicrobial effects of NETs.

Parent and *ΔlptA* Gc were exposed to PMNs that were treated with PMA to release NETs. NETs were either left untreated, treated with DNAse I prior to infection with Gc (NETs+DNAse), or treated with DNAse I after infection with Gc (NETs → DNase). Bacterial survival was calculated by dividing the CFU after 60 min exposure by the CFU at 0 min. *, P<0.05; two-tailed *t*-test, n=3 experiments.

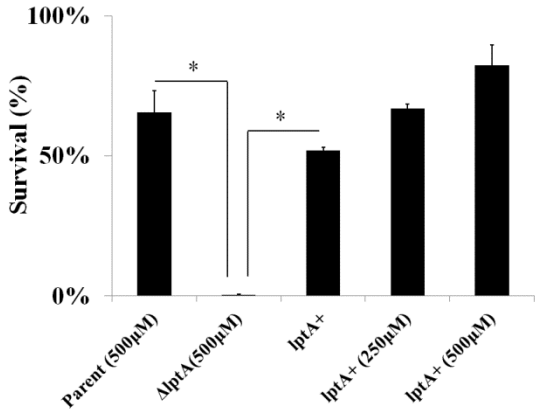

Figure 17. Survival of parent, *lptA* mutant, and *lptA* complemented Gc induced with increasing concentrations of IPTG after exposure to polymyxin B.

Parent, *ΔlptA*, and *lptA*$^{+}$ complement were grown in the presence of IPTG at the indicated concentrations for 2.5 h, then exposed to 2μg ml^{-1}polymyxin B. Survival of Gc after 45 min is expressed as a percent of bacterial CFU at 0 min. *, P<0.05; Student's two-tailed ***t*-**test, n=3 biological replicates per strain.

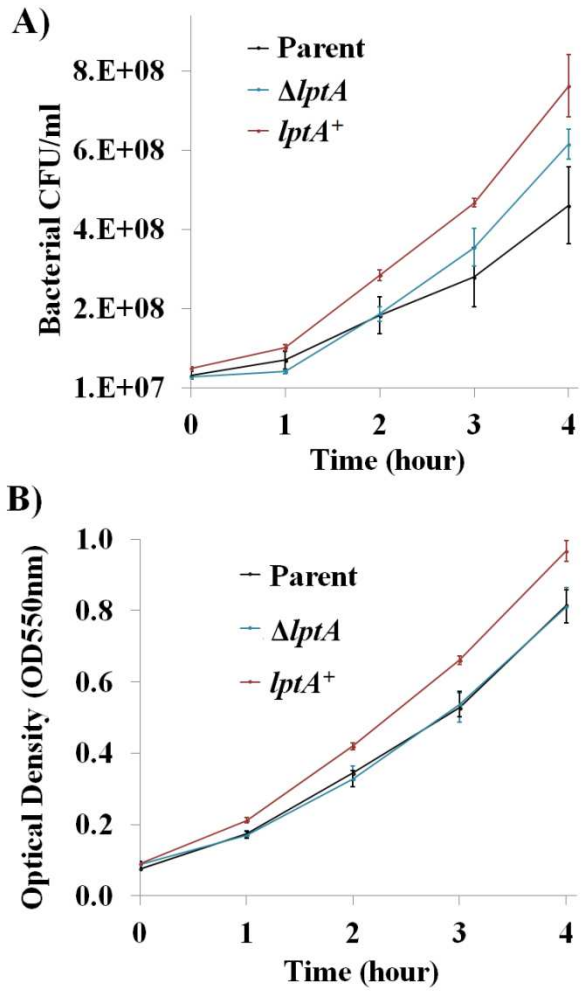

Figure 18. Growth is not attenuated in *lptA* mutant Gc.

Broth-grown parent, *ΔlptA,* and *lptA*$^{+}$ Gc were diluted to an OD_{550} of 0.07 and incubated over 4 h with shaking. Growth was assessed every hour by A) enumeration of CFU and B) optical density (550nm). n=4 biological replicates per strain.

4. Chapter 4: The MtrCDE efflux pump protects *Neisseria gonorrhoeae* within neutrophil extracellular traps and against neutrophil-derived antimicrobial components.

4.1 Introduction

Multi-drug resistance in *Neisseria gonorrhoeae* (Gc) is of grave concern. Gc has developed resistance to all antibiotics recommended for treatment by the US Centers for Disease Control and Prevention (CDC) (CDC, 2011; CDC, 2013). There have been numerous reports of treatment failures with the extended-spectrum cephalosporins (ESCs), which is the last recommended antimicrobial monotherapy for gonorrhea (CDC, 2013). Intermediate resistance to ESCs is conferred by changes in the *penA* gene, encoding the antibiotic target penicillin-binding protein 2 (PBP2). Decreased antibiotic susceptibility has been linked to allelic exchange of the encoded *penA* gene with mosaic alleles, and also to mutations in the β-lactam binding region of PBP2 (Unemo *et al.*, 2012). High ESC resistance can be conferred by further mutations in the *penB* gene, encoding PorB1b, that result in decreased antibiotic uptake, and in the *mtr* system that result in increased antibiotic export due to overexpression of the MtrCDE antimicrobial efflux pump (Warner *et al.*, 2008; Unemo *et al.*, 2012).

The gonococcal multiple transferrable resistance (MtrCDE) efflux pump is highly similar to AcrAB-TolC efflux pump in *Escherichia coli* and MexAB-OprM efflux pump in *Pseudomonas aeruginosa*, which all belong to the hydrophobic and amphiphilic efflux resistance-nodulation-division (HAE-RND) family. The tripartite pump is composed of an inner membrane proton motive force-dependent channel (MtrD) and an outer membrane channel (MtrE), which are connected through a periplasmic membrane fusion protein (MtrC). MtrCDE exports a wide variety of structurally diverse antimicrobial agents including antibiotics, fatty acids, nonionic detergents, bile salts and host-derived cationic peptides (Hagman *et al.*, 1995; Delahay *et al.*, 1997; Shafer *et al.*, 1998). Clinical mutations causing overexpression of MtrCDE occur in either the pump repressor (*mtrR*) or in the promoter region upstream of the *mtrCDE* operon

(Warner *et al.*, 2007). These mutations also confer increased antibiotic resistance of Gc to penicillin, erythromycin and azithromycin (Warner *et al.*, 2007).

In addition to antibiotic resistance, MtrCDE may also be important for Gc survival during infection. Genetic inactivation of the MtrCDE efflux pump results in attenuated survival of Gc *in vivo* in the cervicovaginal murine infection model, while mutations causing derepression of the *mtrCDE* operon that result in increased pump expression can confer fitness benefits to Gc *in vivo* in this same model (Jerse *et al.*, 2003; Warner *et al.*, 2007). This murine infection model involves inoculating estradiol-treated mice with Gc, and results in approximately 80% of the mice are infected for about seven days. In this model, Gc induces release of proinflammatory cytokines that favors recruitment of neutrophils to the vaginal tract. In the host, MtrCDE efflux may protect Gc from the antimicrobial effects of fatty acids and cationic antimicrobial peptides (CAMPs) that bathe mucosal surfaces, including the human CAMP LL-37, a known pump substrate (Shafer *et al.*, 1998).

During infection, Gc resists killing by host-derived antimicrobials in the mucosal layer, but also promotes release of proinflammatory cytokines by the mucosal epithelium that produce a gradient to recruit neutrophils to the site (Waage *et al.*, 1989; Fichorova *et al.*, 2001). Neutrophils mount a robust immune response including phagocytosis of Gc, production of reactive oxygen species (ROS) by NADPH oxidase, formation of neutrophil extracellular traps (NETs) and release of antimicrobial components (Borregaard *et al.*, 2007; Borregaard, 2010; Juneau *et al.*, 2015). However, Gc resists clearance, suggesting that it has virulence factors that mediate defense to neutrophil killing mechanisms (Criss *et al.*, 2009). Neutrophils store high concentrations of antimicrobial components in cytoplasmic granules, such as the LL-37 precursor peptide (hCAP18), other CAMPs and several higher molecular weight cationic antimicrobial proteins (CAPs) (Borregaard *et al.*, 2007). Thus we hypothesize that MtrCDE export of neutrophil-derived antimicrobials is an important part of Gc defense against neutrophils.

Here, we demonstrate that MtrCDE contributes to Gc survival during exposure to neutrophils. We show that MtrCDE improves extracellular, but not intracellular, survival of Gc in two ways. First, MtrCDE efflux protects Gc from degranulated neutrophil-derived antimicrobial components, and second, MtrCDE contributes to Gc defense against the antimicrobial activity of NETs.

4.2 Materials and Methods

4.2.1 *Bacterial strains and growth conditions*

Piliated opacity protein-deficient Gc of strain FA1090 served as the parent for this study ((Ball and Criss, 2013). Gc was maintained on gonococcal medium base (BD Difco) with Kellogg's supplements I + II (GCB) (Kellogg *et al.*, 1963), and routinely grown in rich liquid medium (GCBL) for 16–20 h at 37°C/5% CO2 (Criss and Seifert, 2008).

4.2.2 *Construction of mtr mutant and complement strains*

FA1090 parent Gc was transformed by consecutive backcross with genomic DNA from *mtr* mutants in the FA19 Gc background. Genomic DNA from FA19 *mtrC::kan* or FA19 *mtrD::kan* (a kind gift from Dr. William Shafer, Emory University) was introduced by natural transformation into FA1090 parent Gc, and transformants were selected on 40 μg ml^{-1} kanamycin. Genomic DNA from a kanamycin-resistant transformant was isolated (Qiagen DNA Mini Kit)and retransformed into the FA1090 parent Gc strain, with transformants selected on kanamycin as above, and repeated to yield three consecutive backcrosses in total. To generate the *mtrE* mutant, a PCR product was amplified from genomic DNA of FA19 *mtrE::kan* Gc (from Dr. William Shafer) using the primer pair MTREF (5'-CGA AGA CCA AGG CTT CGT TAT GG-3') and MTRER (5'-AAT ATT CAA TGC CGA CCG GAC C-3') and was introduced by natural transformation into FA1090 parent Gc. Transformants were selected on 40 μg ml^{-1} kanamycin. The FA1090 *mtrC::kan*, *mtrD::kan* and *mtrE::kan* Gc strains were confirmed by PCR and sequencing with

primers specific to each gene that are upstream of the *kan* cassette insertion site: MTRCSEQF (5'- TGC AAC CCG TTC GAA CAT TCG-3'), MTRDSEQF-(5'-AAC GGC GTG GAA GGT TTG G-3') and MTRESEQF (5'-TTG ACC TCT GTT GCA GCA GC-3'). We confirmed that the backcrossed FA1090 *mtrC* and *mtrD* mutants retained the 1-81-S2 *pilE* sequence, using the primer pair: PILRBS (5'-GGC TTT CCC CTT TCA ATT AGG AG-3') SP3A (5'-CCG GAA CGG ACG ACC CCG-3'), and did not express any Opa proteins, as determined by probing bacterial lysates by western blot with a pan-Opa antibody (4B12). Their *pilE* loci were sequenced using the primer pair: PILRBS (5'-GGC TTT CCC CTT TCA ATT AGG AG-3') and SP3A (5'-CCG GAA CGG ACG ACC CCG-3').

To complement FA1090 *mtrE::kan*, Gc were transformed with the pKH35 complementation plasmid (Hamilton *et al.*, 2005) (a kind gift from Joseph Dillard, University of Wisconsin, Madison) containing an IPTG-inducible *mtrE* gene. *mtrE* was amplified from the genomic DNA of FA1090 Parent Gc using the primer pair: MTRE_KpnI_COMPF (5'-TGC AGG TAC CGC AAA ATA CCG TCT GAG AAC C -3') and MTRE_SpeI_COMPR (5'- CAG GAC TAG TCG GTT ATT TGC CGG TTT GG-3'). Empty pKH35 and the PCR product were digested with KpnI and SpeI (New England Biolabs) restriction enzymes, and the PCR product was ligated into the pKH35 multi-cloning site using T4 DNA ligase (New England Biolabs). Successful Gc transformants were selected on 0.5 μg ml^{-1} chloramphenicol, and confirmed by PCR and sequencing using the primer pair: aspC1 (5'-GCC GGA TGC GTC TTT GTA C-3') and lctP (5'-GCG CGA TCG GTG CGT TCT-3'). The *mtrE*$^{+}$ complement was induced with 250 μg ml^{-1} IPTG in rich liquid medium for 2.5 hours before experimental use. At this concentration, the complement showed similar sensitivity to the isogenic parent against the pump substrate LL-37 (Figure 23).

4.2.3 *Neutrophil isolation*

Venous blood from healthy human donors was drawn in heparin tubes, and sedimented on dextran to deplete erythrocytes. The erythrocyte-depleted layer was then purified on a Ficoll-Hypaque

gradient to isolate neutrophils (Stohl *et al.*, 2005). Neutrophils were resuspended in Dulbecco's phosphate buffered saline (PBS; without calcium and magnesium; Thermo Scientific) containing 0.1% dextrose. Neutrophils were kept on ice for no longer than 2 hours before use. Preparations were normally > 95% PMNs. Donors gave informed consent in accordance with a protocol approved by the Virginia Institutional Review Board for Health Sciences Research.

4.2.4 *Neutrophil antimicrobial assay*

Neutrophils (10^6 cells per coverslip) were resuspended in RPMI (Mediatech) containing 10% fetal bovine serum (FBS; Thermo Scientific). Neutrophils were primed with human IL-8 (10 nM; R&D Systems) for 1 hour at 37°C/5% CO_2 to allow adherence to tissue culture-treated plastic coverslips. Adherent neutrophils were infected with exponential phase Gc as described previously (Criss *et al.*, 2009). At indicated time points, neutrophils were lysed in 1% saponin, and Gc was plated on GCB agar.

4.2.5 *Bacterial survival in neutrophil extracellular degranulated supernatant*

Neutrophils (10^6 cells per coverslip) were treated with PMA (10nM; Sigma), and allowed to adhere to tissue culture-treated plastic coverslips for 30 minutes at 37°C/5% CO_2. The supernatant was filtered (0.2 µm) to remove neutrophils. Gc (10^6 CFU) were incubated in 0.2 ml supernatant or RPMI for 1 hour 37°C/5% CO_2. Survival was calculated as the percent of CFU in the treated condition divided by the percent of CFUs in the untreated control.

4.2.6 *Bacterial viability assay*

Baclight viability dyes (Invitrogen) were used in conjunction with lectin SBA Alexa Fluor 647 conjugate (Life Technologies) to discriminate intracellular and extracellular Gc in association with neutrophils as described previously (Criss *et al.*, 2009).

4.2.7 *NET killing assay*

Neutrophils (10^6 cells per coverslip) were treated with PMA (10nM; Sigma), and allowed to adhere to tissue culture-treated plastic coverslips for 30 minutes at 37°C/5% CO_2. Neutrophils were treated with 10 μg ml^{-1} cytochalasin D (Sigma) in the presence or absence of 1 U ml^{-1} DNase I (New England Biolabs) for 20 minutes at 37°C/5% CO_2. Neutrophils were inoculated with Gc at an MOI = 1, and incubated for 1 hour 37°C/5% CO_2. Percent survival was calculated as the fraction of CFU after 1 hour of exposure divided by the CFU at time 0.

4.2.8 *LL-37 antimicrobial susceptibility assay*

Exponential phase Gc (5 x 10^5 CFU) was exposed to LL-37 (from Dr. William Shafer, Emory University) at indicated concentrations in 0.2x GCBL for 1 hour at 37°C/5% CO_2. Survival was calculated as the fraction of CFU at time 1 hour divided by the CFU at time 0 for each concentration, and then normalized to Gc survival in the untreated control (set to 100%).

4.2.9 *Statistics*

Values are the mean ± the standard error of at least three, independent replicates performed on different days. Significance was assessed using a two-tailed Student's *t*-test. A *P*-value of <0.05 was considered significant.

4.3 Results

4.3.1 *MtrCDE contributes to survival of Gc exposed to neutrophils*

Piliated, opacity-protein deficient FA1090 Gc (Parent), three isogenic efflux pump mutants *mtrC*, *mtrD, mtrE* and an *mtrE* mutant complemented with full-length *mtrE* under the control of an isopropyl-β-D- galactosidase (IPTG)-inducible promoter (*mtrE*$^+$) were assessed for survival in the presence of adherent, IL-8 primed human neutrophils (Figure 19). Following infection for 60 minutes, approximately 90% of the parent, *mtrC, mtrD* and *mtrE*$^+$ inocula were recovered, while only 70% of the *mtrE* inoculum

was recovered. At 120 minutes post-infection, the parent, *ΔmtrC, ΔmtrD* and *mtrE*$^{+}$ strains had all recovered to over 135% of inocula, but the *ΔmtrE* strain lagged behind at 105% of inoculum. We were surprised to observe that only deactivation of the outer membrane channel MtrE, but not the inner membrane channel MtrD or the membrane fusion protein MtrC, had a significant impact on Gc survival. These results suggest that MtrE is necessary to completely remove antimicrobials from Gc, and also that the antimicrobials that are pump substrates primarily gain access to the periplasmic space where they are able to be exported by MtrE.

We sought to further evaluate the importance of the MtrCDE pump for intracellular and extracellular survival of Gc exposed to neutrophils. Neutrophils were infected with the parent, *mtrD* or *mtrE* Gc for 1 hour. Gc were treated with fluorescent dyes that discriminate viable and non-viable bacteria based on membrane permeability, and with a fluorescently-labeled lectin to additionally differentiate intracellular and extracellular bacteria (Figure 20A). Both *mtrD* and *mtrE* Gc had decreased extracellular survival compared with the parent, but there was no difference in intracellular survival among the three strains (Figure 20B). There was also no significant difference in internalization of the three strains by neutrophils (Figure 20C). Taken together, these results indicate that MtrCDE efflux protects Gc from extracellular neutrophil killing, and suggest that the pump is either ineffective or unimportant for protecting intracellular Gc from exposure to the higher concentrations of antimicrobial granule components found in degradative phagolysosomes.

4.3.2 *MtrCDE improves Gc resistance to extracellularly released neutrophil-derived antimicrobials*

Since the MtrCDE pump is known to export several host-derived antimicrobials, including the CAMP LL-37, we reasoned that the pump would provide increased resistance to Gc by efflux of antimicrobials released extracellularly by activated neutrophils during infection. To test this hypothesis, we treated neutrophils with PMA to induce exocytosis of all granule types. Neutrophils were removed

by filtration, leaving only the degranulated supernatant containing the antimicrobial granule contents. Parent, *mtrD, mtrE* and *mtrE*$^{+}$ Gc were exposed to the degranulated supernatant for 1 hour (Figure 21). Both *mtrD* and *mtrE* Gc were significantly more susceptible than either parent or *mtrE*$^{+}$ to killing by antimicrobials in the supernatant. Interestingly, Parent and *mtrE*$^{+}$ Gc, which express a functional MtrCDE pump, were completely resistant to the antimicrobials in the supernatant. These results suggest that there are components in the neutrophil-derived supernatant, such as LL-37, to which Gc is susceptible, requiring efflux by MtrCDE.

4.3.3 *MtrCDE protects Gc from the antimicrobial activity of NETs*

Recently, NET formation was observed as part of the innate immune response to infection (Brinkmann *et al.*, 2004). During NET release, neutrophil DNA and histones mix together with antimicrobial granule contents before being released extracellularly to form the characteristic web-like structure. We have shown that Gc induces NET formation by human neutrophils *in vitro* and Gc has defenses against NETs, including release of a nuclease and phosphoethanolamine decoration of its lipooligosaccharide (Handing and Criss, 2015; Juneau *et al.*, 2015). We hypothesized MtrCDE would confer protection to Gc that are immobilized in NETs and exposed to antimicrobial proteins in them, such as LL-37. To test this hypothesis, neutrophils were treated with PMA to induce formation of NETs, followed by treatment with cytochalasin D to block phagocytosis of Gc. To control for NET-specific killing, DNase I was added to half of the conditions to degrade extracellular NET DNA. Parent, *mtrE* and *mtrE*$^{+}$ Gc were exposed to either intact NETs or NETs that had been degraded with DNAse I (Figure 22). Both parent and *mtrE*$^{+}$ Gc were insensitive to NET killing. However, *mtrE* Gc exhibited reduced survival when exposed to NETs, and *mtrE* mutant survival was recovered when exposed to DNAse I-degraded NETs (Figure 22). These results suggest that MtrCDE protects Gc in NETs, potentially by exporting antimicrobials that decorate the NET structure.

4.4 Discussion

Maness and Sparling identified the gonococcal *mtr* system over forty years ago (Maness and Sparling, 1973). Since then, the components of the Mtr efflux system have been examined for their biochemical and structural characteristics and their genetic regulation, and substrates that are exported by the pump have been identified and characterized (Hagman *et al.*, 1995; Delahay *et al.*, 1997; Shafer *et al.*, 1998; Rouquette *et al.*, 1999; Janganan *et al.*, 2011; Zalucki *et al.*, 2012; Janganan *et al.*, 2013). Notably, the Shafer lab elucidated the importance of the efflux pump for export of host-derived antimicrobials including CAMPs and fatty acids (Delahay *et al.*, 1997; Shafer *et al.*, 1998). The Jerse lab has also demonstrated that *mtr* is important for Gc survival *in vivo* in the mouse cervicovaginal infection model (Jerse *et al.*, 2003; Warner *et al.*, 2007; Warner *et al.*, 2008). However, the role of the gonococcal *mtr* system during human infection has not been studied. Neutrophils are abundant at the site of Gc infection in humans, and direct a potent arsenal of antimicrobials to combat Gc. In this study, we show that the MtrCDE efflux pump contributes to extracellular, but not intracellular, survival of Gc exposed to human neutrophils, and does so in two ways. First, MtrCDE protects Gc from killing by antimicrobial granule contents that are released by activated neutrophils. Second, MtrCDE defends Gc from antimicrobial activity mediated by NETs.

The Mtr system is a highly versatile efflux pump that exports a wide variety of structurally-diverse antimicrobial agents. However, many of the pump substrates share amphipathic characteristics. Amphipathic molecules possess regions of hydrophobic and hydrophilic elements that tend to favor interaction with biological membranes. Several of the host-derived antimicrobials exported by MtrCDE such as fatty acids, bile salts, protegrins and LL-37 have amphipathic elements and can interact with bacterial surfaces to promote membrane lysis. Neutrophils store high concentrations of the LL-37 precursor, hCAP18, in secondary granules (Wiesner and Vilcinskas, 2010), which could explain the

importance of MtrCDE for Gc resistance to the degranulated supernatant released from activated neutrophils. However, neutrophils also possess other antimicrobials with amphipathic characteristics including α-defensins (HNPs 1-4), lysozyme, bactericidal/permeability-increasing protein (BPI), CAP37/azurocidin, lactoferrin and cathepsin G. The solved structure of the MtrE outer membrane channel revealed an exit pore of approximately 22 Å in diameter (Lei *et al.*, 2014), which would exclude higher molecular weight proteins such as BPI (55kDa), CAP37 (37kDa) and lactoferrin (80kDa), but could feasibly facilitate export of HNPs (~4kDa), lysozyme (14.5kDa) and cathepsin G (26kDa). In addition, some of the larger neutrophil proteins including lactoferrin are proteolytically processed by host proteases to release active antimicrobial peptide fragments (Wiesner and Vilcinskas, 2010; Sinha *et al.*, 2013), which are within the size range for export by MtrCDE. The evident flexibility of substrate selection by MtrCDE suggests that there are likely many more substrates that have not yet been identified, and some of those could be derived from neutrophils.

NET formation is an important component of the host innate immune response to many bacterial pathogens (Brinkmann *et al.*, 2004). Both Gc and *Neisseria meningitidis* can induce NET release from human neutrophils *in vitro* (Lappann *et al.*, 2013; Juneau *et al.*, 2015). However, they resist killing from the antimicrobial activity mediated by NETs. Gc and *N. meningitidis* modify the lipid A portion of their lipooligosaccharide (LOS) with phosphoethanolamine (PEA), which reduces susceptibility to killing by several neutrophil-derived antimicrobial components, and protects them from killing within NETs (Veal *et al.*, 2002; Hobbs *et al.*, 2013; Handing and Criss, 2015). Several of these antimicrobial components are known to decorate NETs, including LL-37 and cathepsin G (Lappann *et al.*, 2013). We have shown here that the MtrCDE efflux pump also contributes to Gc survival within NET structures. Our observation that MtrCDE is important for Gc defense against a degranulated neutrophil supernatant suggests that the efflux pump also protects Gc from the same neutrophil antimicrobial granule contents that decorate NETs. Another possibility is that MtrCDE is protecting Gc against inherent NET

antimicrobial activity, which occurs through an unknown mechanism, and is independent of the antimicrobial granule contents. Surprisingly, MtrCDE does not have any role in the survival of *N. meningitidis* exposed to NETs (Lappann *et al.*, 2013) (Lappann 2013). *N. meningitidis* may possess additional virulence factors, which are not present in Gc, that are redundant for its protection within NETs. Many *N. meningitidis* strains are encapsulated, whereas all Gc strains are unencapsulated. The meningococcal capsule is significant for defense against several antimicrobials stored in neutrophil granules, including HNP-1, HNP-2 and LL-37, which are also present in NETs (Urban *et al.*, 2009; Lappann *et al.*, 2013).

MtrCDE improves Gc survival in the murine vaginal tract (Jerse *et al.*, 2003), suggesting that the efflux pump may be involved in export of antimicrobials released by host mucosal epithelial cells. We have also shown that MtrCDE protects Gc from extracellular antimicrobials released from human neutrophils, and from those decorating NETs. However, MtrCDE is dispensable for intracellular survival of Gc in neutrophil phagosomes, which contains higher concentrations of antimicrobial granule contents. In addition, the fatty acid resistance efflux pump (FarAB) is dependent on the function of the MtrE outer membrane channel for complete export of toxic fatty acids out of Gc (Lee and Shafer, 1999). Antimicrobial fatty acids may be an unappreciated component of the innate immune response to Gc. Fatty acids bathe mucosal surfaces in the reproductive tract, and there is some evidence that they are also released by neutrophils. Taken together, the *mtr* system is important for protecting Gc during infection by exporting toxic host antimicrobials out of the bacterial cell. Mutations in the *mtr* system that confer increased efflux pump expression are accompanied by fitness benefits during *in vivo* murine infection (Warner *et al.*, 2007). Many of these mutations occur spontaneously in Gc as a resistance mechanism in response to antibiotic therapy. This has severe clinical consequences, but may also be advantageous for Gc during human infection.

4.5 Figures

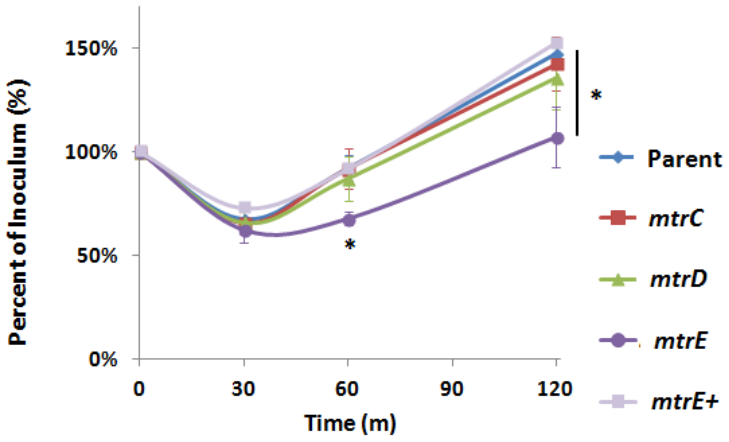

Figure 19. MtrE, but not MtrC or MtrD, is important for survival of Gc exposed to human neutrophils. Adherent neutrophils were infected with parent, *mtrC, mtrD, mtrE* or *mtrE*$^{+}$ Gc. At indicated time points, neutrophils were lysed to free internalized Gc. Survival is expressed as the fraction of CFU at each time point divided by the CFU at time 0. n=7 (n=1 *mtrE*$^{+}$) experiments. *, $P < 0.05$ for parent vs. *mtrE*.

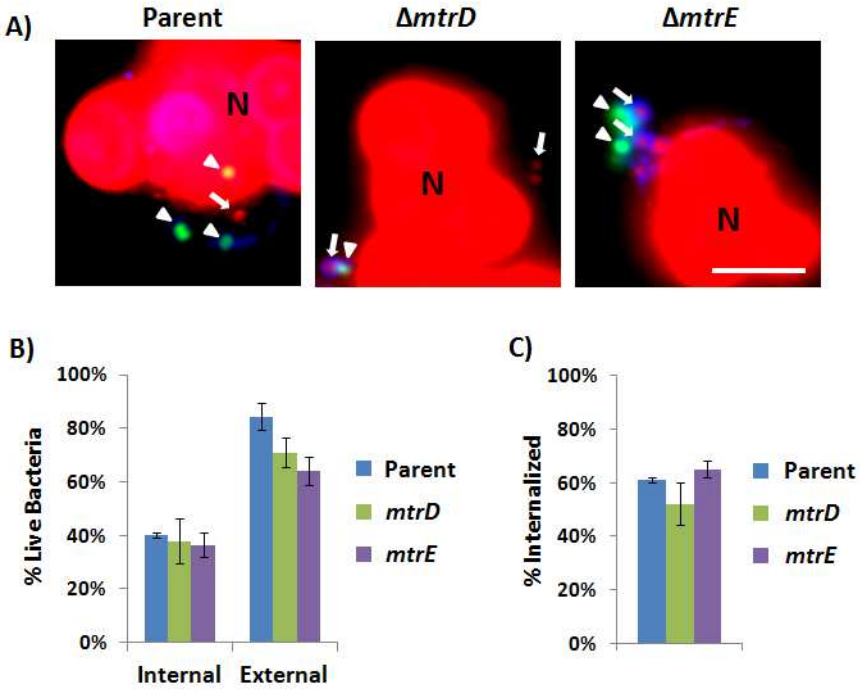

Figure 20. MtrCDE improves survival of extracellular, but intracellular, Gc exposed to neutrophils.

Adherent neutrophils were infected with parent, *mtrD,* or *mtrE* Gc for 1hr. A) Extracellular Gc were stained with lectin SBA Alexa Fluor 647 (blue). Neutrophils were permeabilized, and Gc were stained with Syto9 (green) and propidium iodide (red) to discriminate viable and non-viable Gc, respectively. Arrowheads point to viable bacteria, and arrows to non-viable bacteria. Neutrophil nuclei are propidium iodide positive (N) B) Viable and non-viable Gc were quantified, and each expressed as a percent of the total internal or external Gc for each strain. C) Percent internalization is expressed as the fraction of internal Gc divided by the total number of Gc in association with neutrophils for each strain. n=2 experiments. Scale bar, 5µm.

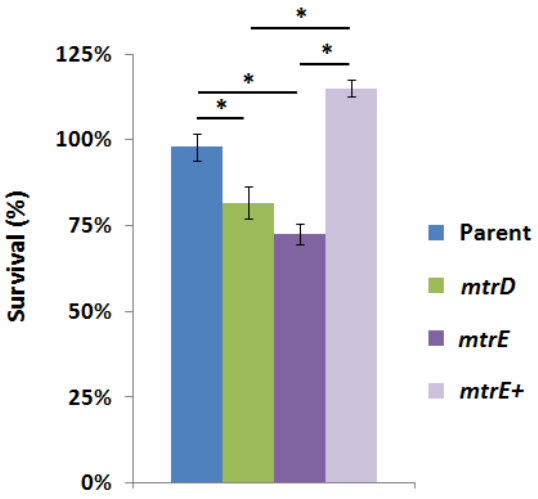

Figure 21. MtrCDE protects Gc from the antimicrobial activity of a degranulated neutrophil supernatant.

Parent, *mtrD, mtrE* or *mtrE*$^{+}$ Gc was exposed to filtered supernatant from PMA-treated neutrophils for 1 hour. Percent survival is expressed as the fraction of CFU in the treated condition divided by the CFU in the no treatment control. n=3 experiments. *, $P < 0.05$.

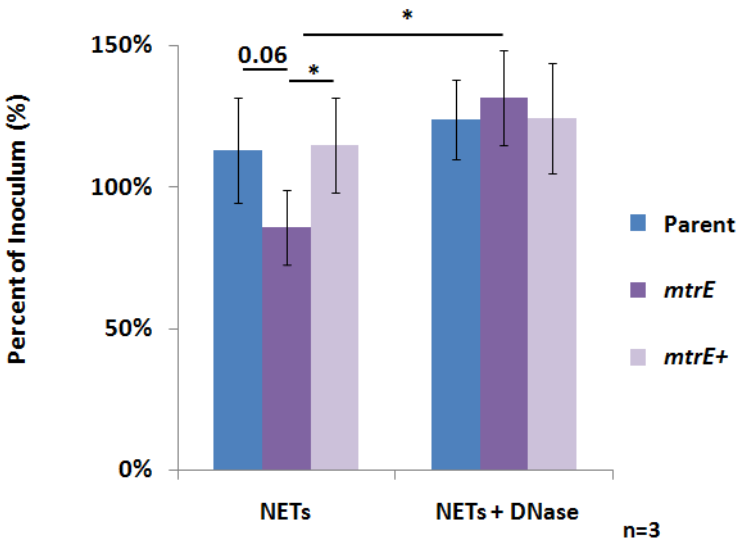

Figure 22. MtrCDE promotes survival of Gc within NETs.

Neutrophils were treated with PMA to promote NET formation. Neutrophils were treated cytochalasin D in the presence or absence of DNase I. Neutrophils were infected with parent, *mtrE* or *mtrE*$^{+}$ Gc for 1 hour. Percent survival is expressed as the fraction of CFU after 1 hour of exposure divided by the CFU at time 0. n=3 experiments. *, $P < 0.05$.

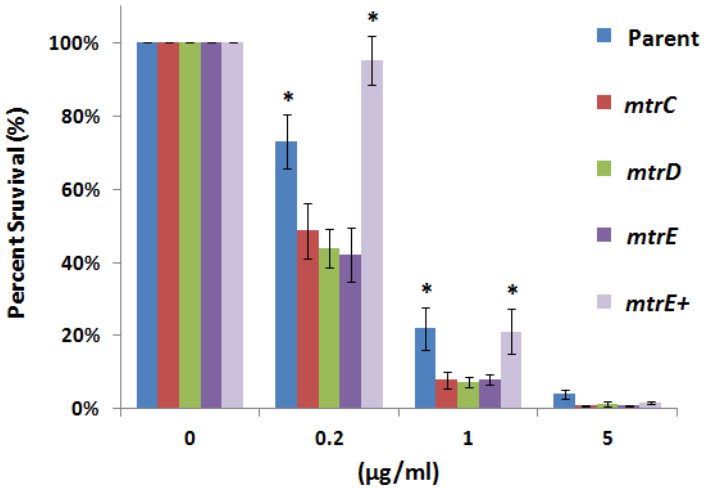

Figure 23. MtrCDE protects Gc from killing by LL-37.

Parent, *mtrC*, *mtrD*, *mtrE* or *mtrE*$^{+}$ Gc were exposed to indicated concentrations of LL-37 for 1 hour. Percent survival is expressed as the fraction of CFU at time 1 hour divided by the CFU at time 0 for each concentration, and then normalized to Gc survival in the untreated control. n=3 experiments. *, $P < 0.05$.

5. Chapter 5: Summary, discussion and future directions

5.1 Summary

In this chapter, I will contextualize my advances to the field, elaborate on Gc interactions with host innate immune defenses and discuss potential paths for future research.

In Chapter 2, I investigated the antigonococcal activity of β-defensin 22 (βD22). Interestingly, βD22 is preferentially expressed in the reproductive tract, which is a major environmental niche for Gc. Prior to this study, several groups showed that βD22 has broad antimicrobial activity against Gram-positive and Gram-negative bacteria as well as fungi (Diao *et al.*, 2011; Liu *et al.*, 2013). However, βD22 had not yet been evaluated for antimicrobial activity against sexually transmitted pathogens. Surprisingly, Gc is susceptible to killing by βD22, despite previous reports of high resistance to both human α- and β-defensins (Qu *et al.*, 1996; Shafer *et al.*, 1998). The βD22 used in this study was a recombinant form of the defensin derived from rat. There are significant sequence differences between human and rat βD22, even within the conserved defensin domain. It is possible that Gc is resistant to the human βD22 as it is with other human β-defensins, but is sensitive to defensins from other species to which Gc is not regularly exposed. Additionally, βD22 differs other defensins, because, in addition to the defensin domain, it has an extended C-terminal lectin domain. I demonstrated that the lectin domain associates with the Gc surface, and could facilitate killing by βD22 during *in vivo* infection. Other defensins may be ineffective at killing Gc, because they lack a lectin domain that allows association with the Gc surface that could more favorably position the defensin domain to interact with and disrupt the bacterial membrane. Notably, I also found that βD22 is active against multidrug-resistant clinical isolates of Gc. These “superbug” isolates are resistant to every currently recommended antibiotic for treatment of gonorrhea. βD22 and similar host-derived antimicrobials may be novel therapeutic options to treat gonorrhea, and could curb the global burden of disease.

In Chapter 3, I investigated the importance of LptA for Gc defense against human neutrophils. Prior to my study, LptA was shown to be important for Gc defense against killing by complement in normal human serum and by CAMPs including polymyxin B and LL-37 (Lewis *et al.*, 2009; Lewis *et al.*, 2013). Neutrophils produce several CAMPs and higher molecular weight CAPs, and are abundant at the site of Gc infection. However, nothing was known about how LptA modification of LOS affected Gc interactions with human neutrophils. Unsurprisingly, I found that LptA protected Gc from several neutrophil-derived cationic components such as HNP-1, CAP37 and cathepsin G. I also showed that LptA protected Gc from NET-mediated killing, which was likely due to the intrinsic defense against neutrophil granule contents that decorate NETs. In addition, I demonstrated that LptA was important for intracellular survival of Gc that had been phagocytosed by neutrophils. Neutrophil phagosomes containing internalized LptA-expressing Gc exhibited reduced fusion with primary granules, but not with other granule types. This was a surprising finding, since LptA also contributes to intrinsic Gc defense against cationic components stored in primary granules. I also observed that LptA protects internalized Gc from serine protease-dependent activity. However, it remains unclear as to whether Gc are protected from direct proteolytic cleavage of surface components that result in bacterial death, or from indirect proteolytic processing of neutrophil-derived precursor proteins that releases mature antimicrobial peptides or signaling molecules that enhance phagocytic killing in some way.

In Chapter 4, I investigated the impact of the MtrCDE efflux pump for survival of Gc exposed to neutrophils. Prior to my study, the Shafer lab demonstrated that MtrCDE has the capacity to export a wide variety of structurally diverse antimicrobials, including many that are host-derived components. The Jerse lab also established that MtrCDE is important for Gc survival during *in vivo* murine cervicovaginal infection (Jerse *et al.*, 2003). The role of MtrCDE during Gc infection in humans had not yet been explored. I observed that the MtrCDE efflux pump does in fact play an important role, by promoting extracellular, but not intracellular, survival of Gc exposed to human neutrophils, and does so

in two ways. First, MtrCDE protects Gc from degranulated neutrophil contents, presumably by export of harmful neutrophil-derived antimicrobials out of the bacterial cell. Second, MtrCDE also improves survival of Gc within NETs, which again is likely due to export of neutrophil-derived antimicrobials that decorate NET structures. Although, it is possible that NET-mediated killing of Gc could be occurring through a mechanism that is independent of antimicrobial granule contents.

5.2 Discussion and future directions

This research represents a significant advancement to our understanding of host-pathogen interactions during human infection with Gc, but there is still much work to be done. In this section, I will talk about potential paths for future research that build off of my own.

Gc is exquisitely adapted to colonize the human host, and, over the millennia, has evolved virulence factors that confer protection against host antimicrobial defenses. Here, I characterized Gc resistance to several host-derived antimicrobials, and showed that LOS-modification by LptA and antimicrobial efflux by MtrCDE protect Gc from antimicrobial neutrophil granule contents. Previously, the Shafer lab described the importance of LptA for Gc resistance to CAMPs, and MtrCDE for the export of structurally diverse hydrophobic antimicrobial agents. The list of known Gc virulence factors that contribute to antimicrobial resistance is incrementally growing, as is the list of antimicrobials to which virulence factors confer protection against. However, we do not have a good understanding of how many of these antimicrobials facilitate their antimicrobial activity, nor do we completely understand the mechanisms by which the virulence factors mediate protection for Gc. To illustrate this point, LptA protects Gc from killing by HNP-1 and CAP37, but not by LL-37, in the FA1090 Gc strain background. All of these are cationic antimicrobials that are thought to interact with the bacterial membrane, yet LptA, which modifies surface LOS, does not provide broad protection against all cationic antimicrobials. Taken together, these results suggest that we need to establish biochemical and biophysical dynamics that

describe these interactions to fully understand the defensive mechanisms of gonococcal virulence factors and the killing mechanisms of host cationic antimicrobials. These research efforts will give context to human infection with Gc, and will inform on the development of novel therapeutic s to combat Gc.

Using host cationic antimicrobials as templates from which to build novel therapeutics may be an appealing idea, but to do so effectively would require a comprehensive understanding of the dynamics involved in bacterial-antimicrobial interactions. I will expand on the potential development of βD22 as a novel therapeutic for treatment of Gc. I found that Gc is highly susceptible to killing by βD22, but is highly resistant to killing by other canonical β-defensins (Qu *et al.*, 1996; Shafer *et al.*, 1998). βD22 is different from these defensins, because it has an extended C-terminal lectin domain. However, I show that the defensin domain of βD22 in necessary and sufficient to kill Gc *in vitro*. β-defensins share a highly similar structural core, but they can differ significantly in their oligomerization and proteolytic stability (Pazgier *et al.*, 2006). These variations could be probed by solving the structure of βD22 either by X-ray crystallography or NMR as a means to characterize its oligomerization pattern, and identify possible structural features that differ from canonical β-defensins. They are also amphipathic peptides that interact with negatively charged bacterial membranes. Several groups have developed artificial membranes and modified bacterial membranes as tools to characterize the bactericidal activity of β-defensins (Pazgier *et al.*, 2006). A modified Gc membrane could be developed based on those methods to characterize the various mechanisms used by βD22, canonical β-defensins as well as by other CAMPs and CAPs to interact with bacterial membranes. Lastly, it has been proposed that the antimicrobial activity and microbe specificity of β-defensins is due to differences in the arrangement of non-conserved amino acid residues (Pazgier *et al.*, 2006). This could be tested by mutating amino acids within these non-conserved regions to probe for the essential residues that confer antigonococcal activity to βD22.

Ideally, βD22 would be passed through a pharmaceutical pipeline as a lead compound for further development and optimization into a therapeutically viable product.

The contribution of LptA to Gc defense is not limited to defense against host-derived antimicrobials. I have shown that LptA-expressing Gc avoid phagosomal fusion with primary granules in neutrophils (Handing and Criss, 2015). LOS modification by LptA could alter recognition of Gc by neutrophil surface receptors, and affect signaling pathways involved in granule mobilization and fusion. LptA catalyzes the addition of PEA to *Neisserial* lipid A. Neutrophils express TLR4, which recognizes lipid A, and PEA-modified lipid A is a more potent activator of TLR4 than unmodified lipid A. *Neisserial* PEA-modified lipid A is highly inflammatory, and induces release of proinflammatory cytokines by monocytes and in the murine genital tract during experimental cervicovaginal infection (John, Liu, and GA Jarvis, 2009; John *et al.*, 2012; Packiam *et al.*, 2014). However, the impact of LOS-modifications for Gc interactions with neutrophils has only begun to be investigated.

In addition to PEA-modification by LptA, Gc LOS can be modified with sialic acid by Lst and with PEA on the inner-core saccharide heptose II by Lpt3 and Lpt6 (Smith *et al.*, 1995; Lewis *et al.*, 2009). Gc that have sialylated LOS are resistant to opsonic and nonopsonic phagocytosis by neutrophils (Wu and Jerse, 2006). The mechanism is not completely understood, but could result from differences in sialylated LOS recognition by neutrophils. Modifications of oligosaccharides on LOS could alter their recognition by lectin and lectin-like receptors on the neutrophil surface, which might differentially activate neutrophils in a way that favors Gc. Different strains of Gc also express natural variants of LOS. These LOS species differ by the length and composition of their oligosaccharide chains, as well as by the number of phosphoryl groups and acyl chains attached to their lipid A (Shafer *et al.*, 2002; John, Liu, and GA Jarvis, 2009; Balthazar *et al.*, 2011). Structural data of LPS in complex with TLR4-CD14-MD2 indicates that lipid A composition strongly influences activation of the receptor (Park *et al.*, 2009). This is

supported by John *et al.*, who demonstrated that the degree of *Neisserial* lipid A phosphorylation, decoration with LptA as well as the number and length of acyl chains dictates the strength of TLR4-CD14-MD2 activation on monocytes (John, Liu, and GA Jarvis, 2009). Differences in phagosome maturation caused by neutrophil infection with LptA-expressing Gc is likely a result of altered recognition of PEA-modified LOS by TLR4-CD14-MD2 on the neutrophil surface. These hypotheses could be tested by inactivating these LOS-modifying enzymes, assessing changes in neutrophil activation following infection with these Gc mutant strains and monitoring changes in downstream signaling pathways. Studies on how Gc LOS affects signaling in neutrophils will be important to understanding the complex interactions of Gc with neutrophils during human infection, and may motivate the development of drugs that target LOS and LOS-modifying enzymes.

Finally, I have shown that MtrCDE protects Gc exposed to neutrophils, but the efflux pump is also clinically relevant because of its involvement in the export of antibiotics. Mutations in the *mtr* system have been identified in multidrug resistant clinical isolates of Gc (Warner *et al.*, 2008). These mutations are located in either the coding sequence of efflux pump repressor gene *(mtrR*) or in the promoter region upstream of the *mtrCDE* operon (Warner *et al.*, 2008). Mutations at either of these loci can result in higher expression of MtrCDE, which confers increased resistance of Gc against killing by antibiotics (Warner *et al.*, 2008). The Jerse lab has shown that antibiotic resistance mutations which cause depression of the MtrCDE efflux pump are accompanied by fitness benefits for Gc during *in vivo* murine cervicovaginal infection (Warner *et al.*, 2007). However, it is not known how these *mtr* mutations affect survival of Gc during human infection. Future work should continue to focus on the clinical consequences of increased MtrCDE expression, but should also focus on how increased MtrCDE expression affects the interactions of Gc with the host innate immune system. However, the primary focus should be on interactions of Gc with the mucosal epithelium and with neutrophils, because of the

importance of MtrCDE for export of host-derived antimicrobials. The results of these studies could better inform therapeutic intervention during disease with multidrug resistant Gc.

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