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Characterization of the Gonococcal Calprotectin Zn-Acquisition System, TdfH, and the Efficacy of Metal Starvation for Treating Gonococcal Disease

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Characterization of the Gonococcal Calprotectin Zn-Acquisition System, TdfH, and the Efficacy of Metal Starvation for Treating Gonococcal Disease.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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List of abbreviations

~	Approximately
<	less than
%	Percent
ºC	degrees Celsius
α	alpha
β	beta
Δ	deletion
ΔH	change in enthalpy
ΔS	change in entropy
Ω	omega
µg	microgram
µL	microliter
µM	micromolar
2D	two-dimensional
3D	three-dimensional
ABC	ATP-binding cassette
AEFI	adverse effects after immunization
AP	Alkaline phosphatase
ASPG-r	asialoglyco-protein receptor
ATP	adenosine tri-phosphate
Az	Azithromycin
BCA	Bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyllphosphate
bp	base pair
BSA	bovine serine albumin
bTf	bovine transferrin

C-	carboxy
$CaCl_2$	Calcium Chloride
CDC	Center for Disease Control and Prevention
CDM	chemically defined chelexed media
CEACAM	carcinoembryonic related cell adhesion molecule
Cef	ceftriaxone
Cip	ciprofloxacin
Cm	chloramphenicol
CMP-NANA	cytidine-5'-monophospho-N-acetylneuraminic acid
CNS	central nervous system
CO_2	carbon dioxide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Fe	iron
Fe^{2+}	ferrous
Fe^{3+}	ferric
$Fe(NO_3)_3$	ferric nitrate
fHbp	factor H binding protein
GCB	GC base medium
H_2O_2	hydrogen peroxide
Hg	hemoglobin
HINT	hydrophobic interactions
HIV	Human Immunodeficiency Virus
Hp	haptoglobin
HRP	horseradish peroxidase
HSPG	heparin sulfate proteoglycans
hTf	human transferrin
IL-10	interleukin 10

IPTG	isopropyl β-D-thiogalactopyranoside
Ka	association constant
Kd	dissociation constant
K_D	affinity
kDa	kiloDalton
KU	Klett unit
L3HΔ	loop 3 helix deletion
L3HA	loop 3 Hemagglutinin insertion
LB	Luria- Bertani *E. coli* growth media
Lf	lactoferrin
LOS	lipooligosaccharide
LPS	lipopolysaccharide
M	molar
MgCl	magnesium chloride
MIC	minimum inhibitory concentration
mL	milliliters
mM	millimolar
mRNA	messenger ribonucleic acid
MSM	men who have sex with men
N-	amino terminus
NANA	N-acetyl neuraminic acid
NaCl	sodium chloride
NadA	*Neisseria* adhesin A
NHBPA	*Neisseria* heparin binding protein A
NBT	nitroblue tetrazolium
Ng	*Neisseria gonorrhoeae*
NHBA	*Neisseria* heparin binding antigen
Nm	*Neisseria meningitidis*

nm	nanometer
nM	nanomolar
OD	optical density
OH-	hydroxide
OH.	hydroxy radical
OMV	outer-membrane vesicle
Opa	opacity protein
PBP	penicillin binding protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDE	phosphodiesterase
PID	pelvic inflammatory disease
polyC	poly-cytosine
polyG	poly Guanine
RBS	ribosome binding site
RNA	ribonucleic acid
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SEM	standard error of the mean
STI	sexually transmitted infection
TBS	tris buffered saline
TdT	TonB-depedent transporter
TGF-β	transforming growth factor beta
Th1	cell-mediated immunity
Th2	humoral immunity
Th17	innate immunity
Tf	transferrin
TfR1	human transferrin receptor

TMB	3,3',5,5'-tetramethylbenzidine
TPEN	TPEN (*N*,*N*,*N′*,*N′*-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine
V	vehicle (DMSO)
WT	Wild Type

Abstract

Characterization of the Gonococcal Calprotectin Zn-Acquisition System, TdfH, and the Efficacy of Metal Starvation for Treating Gonococcal Disease.

By Michael T. Kammerman, B.S.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

Virginia Commonwealth University, 2020

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Neisseria gonorrhoeae, the bacterial agent that is responsible for the human disease gonorrhea, has had a steady increase in the number of infections per year. In 2018 the WHO estimated over 87 million infections occurred world-wide, and the CDC estimated that over 800,000 infections happened in the United States. Accompanying the increase in gonococcal infections per year is the increase in the number of antibiotic resistant isolates being recovered. The recent recovery of a gonococcal isolate that was resistant to the current CDC recommended dual-treatment, coupled with the lack of a preventative vaccine, marks the beginning of an era where there may be no effective treatments for gonococcal disease. The gonococcus possesses a conserved set of proteins that enables it to pirate metal ions from host proteins, and is essential in order for *N. gonorrhoeae* to establish an infection in the human urogenital tract. The importance of TdTs for gonococcal survival and their sequence conservation make them ideal candidates to be included in a gonococcal vaccine, or as targets for potential new therapeutics that are capable of disrupting the interaction with their ligands. In this study we developed a competition assay and probed gonococcal cells with either human (hCP) or mouse calprotectin (mCP) to determine if the gonococcus is species restricted for its ligand interaction. We also performed Isothermal Titration Calorimetry experiments to characterize the binding affinity between the gonococcal calprotectin (CP) binding protein TdfH and calprotectin. In this study we also continued a mutational and small molecule analysis of TbpA aimed at disrupting the interaction with its ligand, human transferrin (hTf). Finally, we investigated if the gonococcal efflux pump, MacA, was responsible for heme export via heme-dependent growth assays of a MacA deficient gonococcal strain. We report that the interaction between TdfH and hCP is high affinity and that the Zn piracy of TdfH occurs optimally at the non-canonical metal binding site of hCP known as

site 1. We determined that multiple mutations, both in the loop 3 helix and in loop 2 of TbpA, minimally reduce the total binding of hTf, similar to what Cash *et al*. has previously described. A screening of a database of small molecules found that several first-generation small molecules were able to significantly reduce the ability of TbpA to interact with hTf. Finally, we found that the efflux pump MacA, does not meaningfully contribute to gonococcal heme export. However, more in-depth studies interrogating MacA substrates are still needed. These studies determined the species specificity of the gonococcal calprotectin binding protein and found that Zn- piracy occurs optimally from one specific site on hCP. This study also shows the promise in investing in new therapeutics that disrupt TdT function.

Chapter 1: Introduction

I. Neisseriaceae

The family of *Neisseriaceae* contains the genera of *Neisseria, Moraxella, Kingella, Acinetobacter,* and *Eikenella*. The organisms within these genera are Gram-negative and have a characteristic oxidase-positive phenotype. The genus *Eikenella* and the genus *Kingella* are predominated by commensal species found in the human oral cavity and bowel. Opportunistic infections by *Kingella* and *Eikenella* species are rare but do occur. *Kingella* species infections occur commonly in bones, joints, and tendons, while *Eikenella* is recovered most commonly from an infection after a being bitten by a human. *Moraxella* species are largely nonpathogenic, with several species considered as parasites of human mucosal surfaces (1). *Moraxella spp.* were once a part of the genus of *Neisseria*. However, studies into *Moraxella* DNA content, fatty acid content, and transformation competence determined that their classification into a separate genus was more appropriate and prompted the transfer of *Moraxella spp.* into the family of *Moraxellaceae* (2-4). The last genus in the family *Neisseriaceae* is the genus *Neisseria*. The human-associated *Neisseria spp.* are often identified by their acid-producing capabilities from glucose or maltose and the ability to reduce nitrite (2). A genomic analysis of the commensal and pathogenic *Neisseria* found a difference in the reductive nitrate capacities been *Neisseria spp.* Only *N. mucosa* was capable of nitrate reduction to nitrite. The ability to reduce nitrite to nitric oxide was a common feature of pathogenic and commensal Neisseria (5).

II. Pathogenic Neisseria

While the majority of *Neisseria* species are commensal bacteria of the nasopharynx, they do have the potential for opportunistic infections (6). Commensal organisms have been recovered from disseminated sites such as blood and CSF despite not being associated with

disease (2,7). Two pathogenic *Neisseria* species are responsible for the majority of the *Neisseria* infections among humans. *Neisseria meningitidis* and *Neisseria gonorrhoeae* are obligate human pathogens that colonize the human nasopharyngeal tract and urogenital tract, respectively. *N. gonorrhoeae* is always pathogenic, and the presence of the gonococcus in the urogenital tract is associated with human disease. *N. meningitidis*, on the other hand, is capable of long-term carriage on the nasopharyngeal tract and results in disease only after invasion into the blood. The gonococcus and meningococcus are non-motile, Gram-negative, catalase-positive diplococci that grow optimally between 35-37^{o}C and require CO_2, which is especially crucial during the early phases of growth (2,8). While there are differences in the epidemiology of these infections, the morphological feature that distinguishes between *N. meningitidis* and *N. gonorrhoeae* is the antiphagocytic polysaccharide capsule produced by *N. meningitidis* (9).

III. Meningococcal Disease

A. Epidemiology

Neisseria meningitidis is associated with carriage and colonization of the human nasopharyngeal tract (10). The carriage rate among the human population is quite variable but can reach upwards of 10-35% in young adults and as high as 80-90% during epidemic outbreaks in Africa (10-12). Transmission of the meningococcus occurs through direct contact with an infected person or their respiratory droplets. In closed or semi-closed environments like military barracks and in-residence college dorms, the meningococcal carriage rate can reach 100% (13). The incidence of meningococcal disease is the highest among infants, with a secondary spike in disease prevalence occurring between adolescence and early adulthood. The incidence of meningococcal disease can vary widely, ranging from 1-1000 cases per 100,000 population/year with the latter number of cases per year being due to localized outbreaks (13,14). The most prevalent occurrences of epidemic meningitis are found within the meningitis belt spanning

across western and central Africa. Epidemic meningococcal disease in the meningitis belt occurs at the end of the dry season, where cases can reach upwards of 1000 cases per 100,000 population (13).

Meningococcal capsular polysaccharides are the basis of molecular serogrouping for the organism. There are 13 different serogroups of *N. meningitidis* with A, B, C, W, and Y being the most frequent causes of invasive human disease worldwide (13,15). The capsular polysaccharides, except for serogroup A, are composed of sialic acid derivatives (14). Serogroup B polysaccharide is composed of sialic acids with α2-8 linkages and is the major cause of disease for Europe, Australia, New Zealand, and the Americas (15,16). Serogroup C polysaccharide connections are composed of α2-9 linkages that are also capable of O-acylation. Serogroup C is prevalent within populations of Asia and the Americas with a minor contribution to disease prevalence in Africa. Serogroup Y is composed of an alternating sequence of D-glucose and O-acylated NANA and can represent a major cause of infections in some countries of the Americas (15,17). Serogroup W polysaccharide only differs from Y polysaccharide by the lack of O-acylation. Serogroup W is the most recent addition to the invasive meningococcal disease group but has spread quickly and is a prevalent cause of infection for Africa and an increasing threat in the Americas (15). Serogroup A is composed of repeating units of (α)-linked *N*-acetyl-mannosamine-1-phosphate, and the prevalence of the disease that it causes is highest in Asia and Africa (15,16).

B. Disease

Meningococci utilize pilin and opacity associated proteins as adhesins for colonization of the nasopharyngeal mucosal epithelium. This binding stimulates the mucosal epithelium to engulf the meningococcal cells, which may then traverse via phagocytic vesicles through

epithelial tissue (18). In a small percentage of colonized individuals, meningococci dissemination into the bloodstream occurs and causes meningococcal disease. Meningitis occurs in patients suffering from meningococcal disease and presents with the classical onset of meningitis symptoms (19). While positive cultures of *N. meningitidis* have been isolated from the blood of patients suffering from meningitis, only in 5-20% of cases does meningococcal sepsis, also known as meningococcemia, occur. Meningitis presents with a rapid onset of symptoms such as headache, stiff neck, photophobia, nausea, and vomiting. Meningococcemia may also present with a petechial rash and hypotension, which can result in multi-organ system failure. Typically, these classical symptoms are seen with young adults and elderly patients. Young children and infants suffering from meningitis present with atypical symptoms, including bulging fontanelle, floppy limbs, lethargy, and high-pitched crying (20,21). Prognosis of meningococcal disease improves with early detection; however, meningitis case fatality rates range from 9-12%, with meningococcemia fatality rates as high as 40%. Within the population of surviving patients, 11-19% can suffer secondary sequelae such as hearing loss, neurologic disability, and loss of a limb (18,21).

C. Treatment and Prevention

Before the availability of antibiotics to treat infectious disease, meningococcal disease reached almost 100% fatality. Over the past few decades, clinicians, through the use of antibiotics, have been able to reduce the fatality rate of meningitis to around 9-12%. Sulfonamides were the first described antibiotics used to treat meningitis in the 1930s'; however, a rise in antimicrobial resistance forced the switch to penicillin, which is still used as the recommended therapy today (18,22). Caretakers and individuals that are in close proximity to an infected person are at an increased risk of infection. Most secondary cases of infection occur

within 5-10 days of exposure, and thus prophylactic antibiotics are prescribed for caretakers and household members of infected individuals to decrease their risk of infection (22). Systemically acting antibiotics that are capable of preventing meningococcal carriage like rifampicin is the optimal prophylactic antibiotic. While antibiotic-resistant meningococcal isolates are rare, they are beginning to emerge (22-25).

Vaccination has been the most effective means of preventing meningococcal disease worldwide. Since the early 1970s, the bivalent (A, C) and a quadrivalent (A, C, W, and Y) polysaccharide vaccine (MSPV4, Sanofi Pasteur) have been used to prevent *N. meningitidis* infection (26). However, the polysaccharide composition of these vaccines produced a T cell-independent immune response, and after the first year of immunization, antibody titers began to decline (27). The polysaccharide only vaccine also did not prevent the future carriage of *N. meningitidis*, which allowed for meningococcal colonization and infection as a person's antibody titer declined if a booster vaccination was not administered (28,29). In 2005, the meningococcal quadrivalent conjugate vaccine (MenACWY, Menactra) was licensed for use in the United States (30). The Serogroup A, C, W, and Y capsular polysaccharide conjugate vaccine allowed for a T cell-dependent immune response resulting in a longer-lasting immune memory than its predecessor and preventing meningococcal carriage (31). The Advisory Committee on Immunization Practices (ACIP) now recommends the MenACWY vaccination as part of routine childhood vaccination with a booster at college age (32,33).

In recent years, *N. meningitidis* serogroup B has accounted for 32% of meningococcal disease reported in the United States and between 45-64% of meningococcal disease in Europe, Australia, and New Zealand (34,35). *N. meningitidis* serogroup B has been difficult to develop a vaccine for due to its polysaccharide structure, which is identical to the polysialic acid found on

many human glycoproteins. The mimicry of human proteins seen with serogroup B capsular polysaccharide makes the use of a polysaccharide-protein conjugate vaccine ineffective (34). To this end, the multivalent outer membrane vesicle (OMV) based vaccine Bexsero (GlaxoSmithKline), was licensed in the United States in 2014 for the prevention of *N. meningitidis* serogroup B disease (36). The 4CMenB vaccine (Bexsero) contains 4 major meningococcal outer-membrane proteins and detoxified outer membrane vesicles (OMV). *N. meningitidis* surface-exposed proteins are highly variable, which necessitates that the chosen antigens be able to induce cross-reactive antibodies for a majority of the invasive meningococcal strains (37-40). The principle antigen of the vaccine is PorA, however, reverse vaccination studies found that the inclusion of *Neisseria* adhesion A (NadA), factor-H binding protein (FHbp) fused with GNA2091, and *Neisseria* heparin binding antigen (NHBA) fused with GNA1030, all presented with detoxified OMVs isolated from the New Zealand outbreak strain NZ98/254 would allow for immunogenicity across strains (35,36). Trumemba® (Pfizer) is also a Serogroup B vaccine that uses the FHbp variants (A05 and B01) and was licensed for use in 2014 in the United States (35). The 4CMenB vaccination has not been associated with any significant safety concerns, and the majority of adverse reactions relates to local reactions (41%) or fever (40%) (41). The 4CMenB vaccine can be administered concomitantly with other routine vaccinations, but the current US recommendation is vaccination with 4CMenB at age 16 (42) and is not associated with an increased risk of adverse effects after immunization (AEFI) (43,44). The implementation of the group B vaccine is a promising move forward; however, further studies at monitoring the long-term efficacy of these vaccines for their decline in antibody titer and AEFI are still required.

IV. Gonococcal Disease

A. Epidemiology

The second of the two pathogenic *Neisseria spp.* is *Neisseria gonorrhoeae*, which is the bacterium responsible for the sexually transmitted infection (STI) gonorrhea (45). *N. gonorrhoeae* infections have steadily increased worldwide, with the World Health Organization (WHO) estimating over 86.9 million cases globally, and 583,000 reported cases in the United States. Gonorrhea is the second most commonly-reported infectious disease in the U.S. (45). *N. gonorrhoeae* is an obligate human pathogen and predominantly spread through direct sexual contact. Factors such as age, race, sexual orientation, and socio-economic status play significant roles in the spread of the disease. Individuals between the age of 15-29 are at the highest risk for contracting gonorrhea, and that risk is also influenced by the number of sexual partners an individual has (45). Individuals of African descent and Native Americans accounted for the largest demographic of infected individuals in 2018. In populations of men who have sex with men (MSM), infection rates continue to rise, and there is an increased risk associated with HIV infections due to the increase in HIV viral replication seen with gonococcal coinfection (45-48). A disparity between the number of asymptomatic infections and the reported infection rates of men and women has also been documented. There are more reported cases of *N. gonorrhoeae* infections in men compared to women. However, over 50% of infections in women are asymptomatic and, thus women often do not seek treatment. The asymptomatic nature of female infection is believed to make women important reservoirs of *N. gonorrhoeae* and contribute to increased morbidities such as pelvic inflammatory disease (PID), ectopic pregnancy, and infertility as well as the continued spread of the bacterium within the population (48,49).

B. Disease

Primary infections with *N. gonorrhoeae* can result in urogenital, anorectal, pharyngeal, and conjunctival infections (50). Most men infected with *N. gonorrhoeae* present with symptoms, including dysuria and urethritis. In 10% of cases, men are asymptomatic, which can result in an ascending infection manifesting as prostatitis and epididymitis (51). Infections in women typically present as cervicitis manifesting 5-10 days post-infection, but over 50% of women are asymptomatically infected (52). Since asymptomatic infections remain untreated serious secondary sequelae can emerge. The infection can ascend the reproductive tract resulting in pelvic inflammatory disease, salpingitis, and damage to the fallopian tubes that can result in an ectopic pregnancy (51). Pharyngeal infections in both men and women are asymptomatic in 90% of cases and are more difficult to diagnose and treat compared to urogenital infections (53,54). Rectal infections are also difficult to diagnose due to them commonly being asymptomatic but can be treated with antibiotics as effectively as urogenital infections. Symptoms of rectal infections can vary dramatically from mild discharge and itching to overt proctitis (55).

The lack of a protective immune response after *N. gonorrhoeae* infection is a hallmark of disease and allows for repeated infections of a person with the same strain of *N. gonorrhoeae*. Interestingly, the gonococcus has been documented to upregulate Th-17 and T-regulatory responses, skewing the immune system away from a protective Th-1 or Th-2 responses (56,57). Gonococcal promotion of a Th-17 response recruits polymorphonuclear cells (PMN) to the site of infection, which is an innate response that is more to the gonococcus benefit. The gonococcus is able to survive inside these recruited PMNs, and survive oxidative bursts generated by PMNs, which makes these recruited PMN ineffective at controlling gonococcal infections (58).

C. Treatment

N. gonorrhoeae has shown a remarkable ability to gain resistance to antibiotics, which began in the early 1930s when sulfonamides were first prescribed to treat gonococcal infections. Within 10 years of sulfonamide implementation, highly-resistant strains had begun to emerge, which prompted the shift by clinicians to prescribe penicillin for treatment (59). Since that time, the steady increase in antimicrobial resistance (AMR) in gonococcal isolates slowly chipped away at clinically-useful antibiotics intended to treat gonococcal disease (60). The WHO and Centers for Disease Control and Prevention (CDC) recommend that treatment options be accessible to all populations and have a 95% cure rate when given as a single dose (45,59). In 2014 the CDC removed fluoroquinolones as a recommended treatment for gonococcal infection, leaving only a few clinically-useful antibiotics. The current CDC-recommended treatment is dual therapy of 250 mg of intramuscular ceftriaxone and 1 g oral azithromycin. This treatment is in line with the ability to effectively treat pharyngeal gonococcal infections and chlamydial coinfections (61,62). A recent case involving a patient infected by a strain exhibiting high levels of resistance to ceftriaxone and azithromycin marks the beginning of an era where no clinically viable treatment for gonococcal disease may exist (61). Given the emergence of high levels of resistance to third-generation cephalosporins, specifically ceftriaxone, studies investigating alternative therapies have been conducted. The *in vitro* synergy of either gentamicin/ azithromycin or gemifloxacin/azithromycin were found to effectively clear urogenital, pharyngeal, and rectal infections with minimal adverse effects post-treatment (63). It is a top priority of the WHO to reduce the global incidence of ceftriaxone resistance in order to maintain a viable treatment for gonococcal disease. The combined issue of the lack of a protective

immune response and dwindling treatment options makes it critical for future investment into new therapies and treatments for this infection.

V. *N. gonorrhoeae* Virulence Factors

N. gonorrhoeae possesses an array of virulence factors that assist in adherence, infection, and immune evasion (**Figure 1**). On top of the numerous gonococcal surface structures, the regulation of these structures plays an important role in immune invasion. Many of these surface structures are subject to phase variation, acting to modify protein expression and protein level. Further, gonococcal surface structures are highly variable, enabling immune invasion.

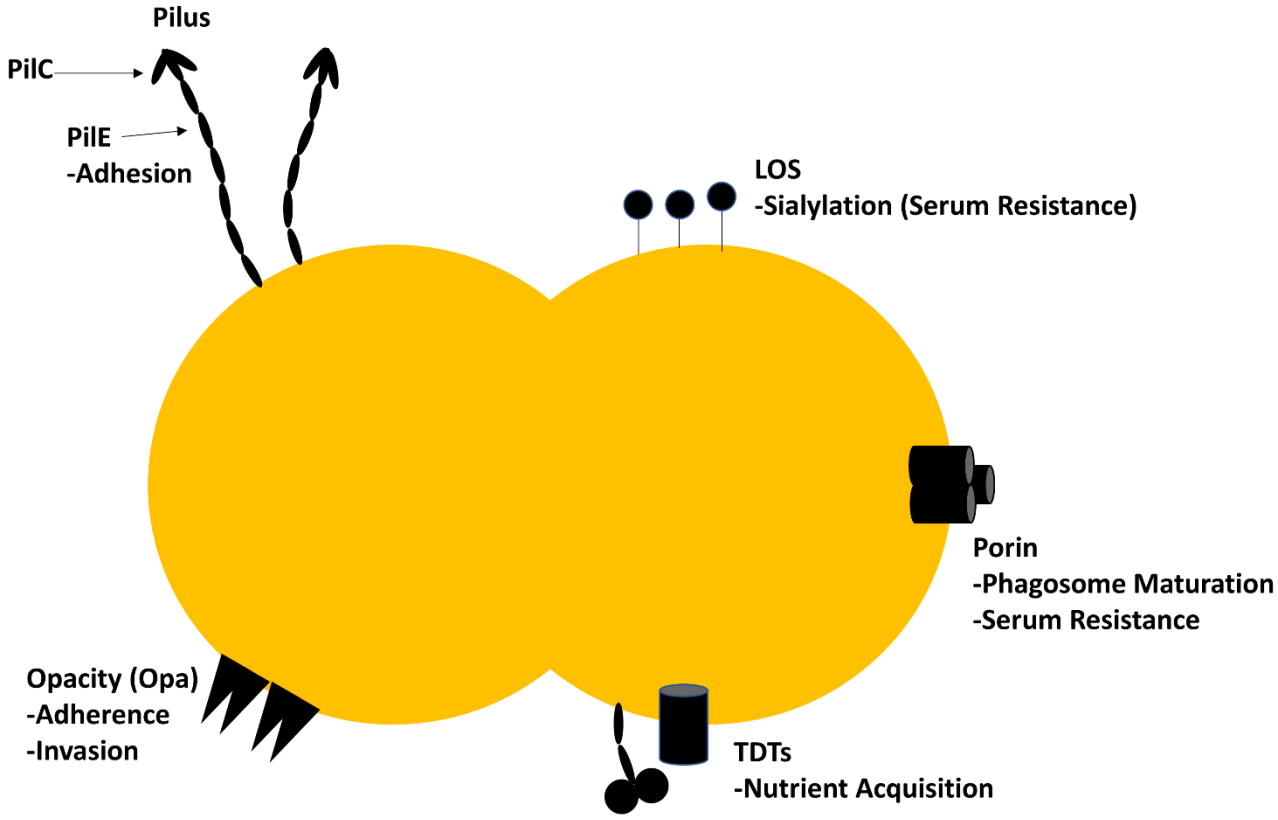

Figure 1. Overview of Gonococcal Virulence Factors

Figure 1.) Overview of *N. gonorrhoeae* Virulence Factors. Image of a gonococcal cell depicting several surface exposed virulence factors expressed by *N. gonorrhoeae*. Image adapted from (64)

A. Type IV Pilus

Neisserial type IV pili are hair-like extensions that are approximately 6 nm in diameter and can extend for several micrometers away from the diplococcus (65). The type IV pilus is essential to infection and allows for the colonization of mucosal surfaces. Gonococcal pilin synthesis requires 23 genes and occurs in 4 steps: assembly, functional maturation, counter-retraction, and emergence on the cell surface (66,67). PilE is an 18-22 kDa protein and is the major structural component of gonococcal pilin that assembles into a helix to generate the pilus structure. Adhesion to cervical epithelial tissue and microcolony formation is mediated by these assembled pili (66). The minor pilin proteins PilC, PilV, and PilX, can also be incorporated into the mature pilus structure; these minor pilins modulate the function of the type IV pilus (68,69). The pilus of *N. gonorrhoeae* undergoes high-frequency antigenic variation through non-reciprocal gene conversion. Non-reciprocal gene conversion is RecA-dependent and occurs between *pilS* (silent genes) with the *pilE* structural gene, resulting in an antigenically new pilin protein. The gene conversion of *pilE* happens at a frequency of 10^{-2} cells (70,71). PilE is also subject to high-frequency phase variation through a polyC tract found within *pilE*. Slip-strand mispairing results in frameshifts that can generate early stop codons, which alter gonococcal pilus production. PilE is also subject to post-translational modifications such as glycosylation, which further modulates pilin function during infection (72). *N. gonorrhoeae* pilin protein is a multi-functional protein, as it is not only necessary for adhesion to host cells during infection but has also been found to bind extracellular free DNA (73,74). The DNA binding of the gonococcal pilus has a connection to the natural competency and transformation ability that is characteristic of *Neisseria* species (66).

B. Lipooligosaccharide (LOS)

Lipopolysaccharide (LPS) is a common feature of the outer membrane of Gram-negative bacteria. LPS is composed of three moieties, lipid A, a core polysaccharide, and O antigen. Lipid A acts as the outer-membrane anchor for LPS, the core polysaccharide is a short oligosaccharide motif, and O-antigen is a variable-length polysaccharide that extends away from the bacteria (75). *N. gonorrhoeae* LPS lacks the variable-length O-antigen, unlike LPS of enteric bacteria, and is often referred to as lipooligosaccharide (LOS). LOS of *N. gonorrhoeae* is also able to antigenically vary itself at high frequency through the phase-variability of glycosyltransferases that are responsible for catalyzing the extension of the LOS carbohydrate chain (76). Three such glycosyltransferases, *lgtA*, *lgtC*, and *lgtD*, have polyG tracts that allow for slip-strand mispairing during DNA replication (77).

The antigenic variability of LOS is just one way that the gonococcus can subvert immune detection. LOS undergoes sialylation through host cytidine5′-monophospho-N-acetyl neuraminic acid (CMP-NANA). Sialylation of gonococcal LOS allows for host Factor H binding protein (FHbp) deposition and confers serum resistance to the bacteria. However, the sialylation of LOS only occurs with certain variants and is considered phase variable due to the phase variability of the glycosyltransferases responsible for extending residues capable of being sialylated (78,79). Interestingly, the sialylation of gonococcal LOS interferes with gonococcal invasion of certain cell types. Invasion through epithelial tissue may promote the population of cells that become serum resistant through the sialylation of specific LOS variants and provides an explanation for the phase variable nature of LOS (80).

The LOS structure of *N. gonorrhoeae* is immunochemically identical to various human glycosphingolipids and glycolipids (81). Gonococcal LOS that mimics paragloboside is also

capable of binding to asialyoglycoprotein receptor (ASP-R) found in the male urethral epithelium and promotes intracellular invasion. The invasion of male urethral cells by the gonococcus is predicted to aid in disease spread due to the presence of ASP-R positive urethral cells found in urethral exudate (82,83). A link between heptose derived from gonococcal LOS and an increase in HIV-1 viral replication underscores the public health importance that this pathogen has for other diseases pathology (84).

In addition to LOS sialylation, neisserial LOS has been shown to be decorated with phosphoethanolamine (PEA) via the PEA Transferase A [*lptA,* (85)]. PEA decoration of gonococcal LOS has been found to provide the gonococcus resistance to the bacterial derived cationic antimicrobial peptide (CAMP) polymyxin B, specifically when PEA is catalytically added to the 4' position of gonococcal lipid A (85). The presence of PEA modification of LOS has been associated with resistance to complement mediated killing when found within the gonococcus but not the meningococcus (86,87). Further, the presence of PEA decorated LOS increases the induction of proinflammatory cytokines, such as TNF-α, which is driven by Toll-like receptor 4 (TLR4) signaling (88). PEA decoration of gonococcal LOS has been shown to have a dual immunostimulatory and protective role during infection. Gonococcal cells that were unable to express *lptA* were at a competitive disadvantage during a genital tract infection of Balb/C mice, and demonstrates that the importance that PEA decoration of LOS has during infection (89). Interestingly, *lptA* was reported to only be found in the genome of the pathogenic *Neisseria* and *N. lactamica*, a commensal *Neisseria* (90). The absence of the *lptA* gene in the genome of the commensal *Neisseria* has been hypothesized as a mechanism that has allows for commensals to remain within the host without inducing the protective host response (90).

C. Porin

Porin is a major component of the gonococcal outer-membrane constituting about 60% of the outer membrane total protein content (91). Neisserial porins form trimeric complexes in the outer membrane. Each monomer forms a β-barrel in the outer membrane (92,93). Porins are essential to the survival of *Neisseria spp*. and function as pores that allow ions and small nutrients across the membrane (94). *N. meningitidis* encodes two porins in its genome. Class 1 porin is commonly referred to as PorA and is the larger of the two porins at around 45 kDa and phase variable. Class 2 and 3 porins, or PorB, is smaller at around 33-35 kDa (95,96). *N. gonorrhoeae* PorA exists as a pseudogene due to mutations in the promoter and coding regions and thus is only capable of expressing PorB (94,97). Gonococcal PorB can be further classified into two subgroups of protein 1A (PorB1A) and protein 1B (PorB1B) (94).

Por1A is associated with an invasive phenotype and increased resistance to killing by complement. The expression of Por1A may be associated with a tendency to cause disseminated infection based on epidemiological evidence (98). Additionally, gonococcal porins can aid in pathogenesis through the insertion into host cell membranes. This insertion results in a pore within eukaryotic cell membrane that changes the charge of the cell membrane interfering with host cell signaling (99). Gonococcal porins inserted into the membrane of the phagosome prevent their degranulation and maturation, independent of NADPH activity. The lack of maturation is thought to promote intracellular survival of *N. gonorrhoeae* that have been phagocytosed by neutrophils and macrophages (100,101).

D. Opacity (Opa) Proteins

Opacity (Opa) proteins are a family of outer membrane proteins that mediate tight adherence and transcytosis of epithelial cells and leukocytes (102,103). Opa proteins consist of 8 transmembrane domains and 4 surface-exposed loops, which contain two hyper-variable regions and a semi-variable domain. The hyper-variable region and semi-variable domain increase the number of unique Opa alleles that *N. gonorrhoeae* or *N. meningitidis* can express through intergenic and interstrain recombination of Opa proteins (65,104). The number of opacity proteins encoded in the genome of *N. gonorrhoeae* and *N. meningitidis* differs, with the gonococcus encoding ~11 different Opa proteins and the meningococcus encoding between 4 and 5 different Opa proteins. Every Opa gene sequenced to date contains a pentameric pyrimidine tandem repeat $(CTCTT)_n$ that is present within the 5'-region of the signal peptide coding sequence for gonococcal and meningococcal Opa proteins. These pentameric repeats allows for high-frequency phase variation via slipped-strand mispairing resulting in a heterogeneous population of cells expressing none, one, or multiple different Opa proteins (105,106).

The critical functions of the Opa proteins are highlighted by their expression during both experimental and natural infections (107,108). An experimental infection study of human male volunteers found that the initial inoculum of mostly Opa negative cells was recovered as Opa positive, indicating a strong selective pressure for Opa expression during infection (109). Opa proteins have been found to play a multi-factorial role during gonococcal and meningococcal adherence and invasion, and the compliment of Opa proteins expressed is the ultimate determinant of these roles (110). Opa proteins allow for the tight adherence to multiple cell types including epithelial cells and various immune cells (111). Opa proteins have a receptor tropism

for carcinoembryonic antigen cell adhesion molecule (CEACAM) and heparin sulfate proteoglycans (HSPG), which are responsible for the neisserial range of cellular adherence and disease pathology (112-116). Opa-CEACAM5 interaction on vaginal epithelia promotes long term lower-genital tract colonization. However, the interaction of Opa-CEACAM 1 on uterine epithelia enhances gonococcal penetration of the tissue (117). Despite the positive benefit that Opa-CEACAM interactions have for *N. gonorrhoeae*, Opa binding to CEACAM 3 expressed on the surface of neutrophils enhances their phagocytosis and killing. Thus, the Opa variants present within the infecting population and the population of CEACAM expressing cells within the infected tissue greatly determines the outcome of infection (117-119).

Beyond the multi-factorial adhesion properties of Opa proteins, they also provide the gonococcus with the ability to modify the immune response to prevent a protective response. Opa 1 interaction with B-cells expressing CEACAM 1 induces cellular death of those B-cells and results in lower overall antibody production during gonococcal infection (120). Similarly, gonococcal cells can selectively suppress CD4+ T- lymphocytes activation and proliferation through the interaction of Opa 52 with CEACAM 1 (121). In addition to the cellular inhibition mediated by gonococcal Opa proteins, they can also manipulate the immune environment by interaction with lymphocytic CEACAMS. Opa mediated interaction of lymphocytes induces the production of TGF-β and IL-10, allowing for suppression of a protective Th-1/Th-2 response and promotes a Th-17 immune response, which is ultimately to the benefit of the gonococcus (56,57,122).

VI. Iron Sources in Humans

Iron is essential for life with very few exceptions and, as such, iron acquisition is recognized as a key step in bacterial pathogenesis. Iron exists in a readily interchange redox

state, which is either a ferrous (Fe^{2+}) or ferric (Fe^{3+}) form. The redox potential, ranging from -300 mV to +700 mV, and the abundance of iron during the early evolution of life made it an ideal candidate for the incorporation into proteins as biocatalysts and electron carriers. Iron's biological functions are completely dependent on its incorporation into proteins or more complex structures such as iron-sulfur clusters or heme groups (123). The chemical properties that make iron ideal for biological processes also allow for potentially harmful and toxic effects. In aerobic environments, iron is found predominantly as Fe^{3+} and has a solubility at pH 7 of 1.4 X 10^{-9} M (124). The relative insolubility of ferric iron in the presence of oxygen necessitates the reduction to its ferrous form. The reduced ferrous form of iron potentiates iron toxicity through its participation in Fenton Reactions ($Fe^{2+}+H_2O_2 \rightarrow Fe^{3+}+OH^{\bullet}+OH^-$) and produces hydroxy radicals that can damage DNA, proteins, and lipids (125).

The aerobic environment of the human body contains ~3.5g of iron, which requires tight regulation for protection against toxicity (126,127). The sequestration of iron is achieved intracellularly by ferritin and extracellularly by iron-scavenging or transport proteins (124). Iron storage proteins leave the human body almost devoid of iron, with free iron levels in plasma being ~10^{-18} M (128). These mechanisms for iron sequestration also provide a counter-measure of defense against invading pathogens. The lack of free iron found within the human body makes it a hostile environment for bacteria, and the host iron-withholding defenses to these bacteria are referred to as nutritional immunity (129). In response to invading microorganisms, intestinal assimilation of dietary iron is reduced, and there is an increased production of iron-withholding proteins that reduces the free iron levels in the body (130). The ability of a pathogen to acquire iron and overcome host nutritional immunity is therefore a critical virulence determinant for the

initiation and continuation of infection (130,131). In the absence of available free iron during infection, host iron binding proteins act as the bioavailable pool of iron for these microbes. Described below is a summary description of the iron binding proteins of humans.

A. Transferrin

Human transferrin (hTf) is an 80 kDa glycosylated protein that is synthesized in the liver and secreted into blood plasma (132). There are two lobes of hTf (N-lobe and C-lobe) believed to have been generated by an ancestral gene duplication (133). The N- and C- lobe of hTf are both capable of binding to Fe^{3+} with an approximate K_D of 10^{-22} M (134). Circulating hTf is approximately 30% saturated with Fe^{3+}, but that can vary depending on the availability of dietary iron and the infection status of a person. The serum concentration can also range widely from 25-50 μM (135). At any point in time, 4 different species of Fe^{3+} loaded hTf can exist in circulation, a monoferric N-lobe, monoferric C-lobe, diferric-hTf (both N and C-lobe have bound to Fe^{3+}), or apo-hTf (136,137). The N-lobe seems to be bound by a Fe^{3+} ion at a higher frequency then the C-lobe; however, no clear distinction as to the reason or relevance *in vivo* has been determined (136,138). All cells that require iron express human transferrin receptor (TFR1) on their surface. TFR1 interacts with diferric hTf with the highest affinity (~4 nM) at pH 7.4, but both monoferric forms of hTf do form a high affinity stable complex with TFR1 (~30 nM) at pH 7.4 (139). Internalization of the TFR1-hTf complex occurs through clathrin-dependent endocytosis leading to the acidification of the endosome and release of Fe^{3+} (140). Apo-hTf has its highest affinity for TFR1 at low pH, which allows for the recycling of hTf back into the serum through displacement by an iron-containing hTf or its dissociation from TFR1 (141).

B. Lactoferrin

Human lactoferrin (hLf) is an 80 kDa glycosylated iron-binding protein that belongs to the transferrin protein family. Similar to transferrin, hLf is bilobed, but the binding site of each lobe binds to a Fe^{3+} ion with high affinity and to a CO_3^{2+} ion. Other metals, such as Cu^{2+}, Zn^{2+}, and Mn^{2+}, have also been documented for their capability to bind to lactoferrin (142). Lactoferrin is found within mucosal secretions such as saliva, tears, semen, vaginal secretions, and is the second most abundant protein in milk (143). Serum concentrations of hLf are low (3.8-8.8 nM), compared to the levels found within mucosal secretions (6-13 μM)(142,144) Neutrophil secondary granules contain high concentrations of hLf, and this is proposed to have a significant physiological role during inflammation (145). The capability of hLf to remain bound to iron over a wide pH range makes it unique among the transferrin protein family (146). During infection, at local sites of inflammation, hLf levels can reach 200 μg/mL, and aside from its role in iron homeostasis, hLf has also been documented to be antimicrobial against a broad-spectrum of pathogenic organisms (147,148). The antimicrobial effects of lactoferrin have been attributed to two mechanisms with the first being its metal withholding properties. The second is the ability of cationic peptide formation after proteolytic cleavage of lactoferrin to lactoferricin B. (149,150). Lactoferricin B has an overall positive charge which allows for interaction with the negatively charged membrane of pathogens and their associated surface structures like LPS or LTA. The membrane association of lactoferricin B results in membrane destabilization and enhances other innate effectors like lysozyme, which results in the death of the pathogen (151,152).

C. Ferritin

When intracellular iron levels are in excess, the potential noxious chemistry is mitigated by the deployment of ferritins. Ferritin is composed of a 24 subunit heteropolymer of two different

chains, which are the heavy (H) chain (210 kDa) and light (L) chain (195 kDa). The H and L chains are both required for the formation of functional, mature ferritin resulting in a spherical shell which is approximately 450 kDa. The ferritin shell accommodates up to 4500 oxygen and hydroxyl-bridged iron atoms, though most ferritins that have been isolated contain between 200 and 2500 iron atoms. Ferritins are highly conserved throughout all of life and the need for ferritins can be demonstrated by the lethality that a ferritin knockout (KO) has in mice (153,154). Proteasomal degradation of ferritin liberates the sequestered iron and is thought to provide iron to the cell as available iron begins to dwindle (155). Hemosiderin is a water-insoluble, degradation product of ferritin. Hemosiderin's core is more heterogenous then that of ferritin and allows for a slower release of iron, but iron release from hemosiderin does occur under acidic conditions (156,157).

D. Heme/Hemoproteins

Approximately 70% of the human body's iron is stored as heme, which is a heterocyclic ring capable of coordinating one ferric iron atom (158). The heterocyclic ring, also known as protoporphyrin, is critical for cellular respiration, enzymatic reactions and oxygen transport throughout the body. Heme is synthesized in a majority of human cell types and can also potentiate toxicity through the creation of reactive oxygen species (ROS) and lipid peroxidation (159,160). Given the potential of heme toxicity to cells, 95% of heme is bound to various heme proteins. The most common hemoprotein is hemoglobin and it accounts for ~65% of the total hemoprotein in the human body and transported around via erythrocytes (158). Hemoglobin (Hg) is a tetrameric protein that consists of two α-chains and two β-chains. Each subunit of Hg binds to a molecule of heme, which in turn binds to oxygen and allows for oxygen transport. While much of the heme is found intracellularly, spontaneous hemolysis of red blood cells

causes the release of Hg, where serum concentrations can range from 8-800 nM (144). Hemoglobin released through hemolysis can be bound by haptoglobin for eventual recycling by macrophages or in the liver (161).

E. Siderophores

Siderophores are low-molecular-weight (less than 1 kDa) molecules that are secreted and utilized by microbes to overcome the iron-limited conditions of the host or the environment (162). Siderophores have high specificity, and affinity for Fe^{3+}. Strong affinities, ranging from 10^{-22} to 10^{-52} M, is enough to remove Fe^{3+} from host proteins such as ferritin, hTf, and hLf (124,163). Siderophores are generally produced by large multi-enzyme synthetases that create a high degree of structural variability in the iron-coordinating residues and can be classified into one of three categories: catecholate, hydroxamate, and hydroxycarboxylate (162,164). Siderophores are secreted into the extracellular environment where they scavenge available iron. Iron loaded siderophores can deliver their iron cargo to microorganisms through siderophore specific receptors. Some microorganisms co-opt xenosiderophores produced by other bacteria and therefore have a selective advantage for iron acquisition within a population (164).

In response to siderophore mediated iron piracy, mammalian hosts have evolved an immune counter-measure: that is, to sequester ferric-siderophores complexes away from their respective siderophore receptors. These siderophore sequestering proteins belong to the lipocalin family of binding proteins and are commonly referred to as siderocalins (165). Siderocalins bind to catecholate-type siderophores, such as enterobactin, with sub-nanomolar affinity and sequester them so that they are unavailable for bacterial use (165,166). Siderocalin KO mice show a significant increase in susceptibility to bacterial infection for bacteria that rely on siderophore mediated iron acquisition (167-169). In response to siderocalins bacteria have evolved modified

siderophores that sterically hinder siderocalin binding. Pathogenic *E. coli* and *S. typhimurium* both produce salmochelin, a C-glycosylated enterobactin analog, encoded by a five gene *iroA* locus. *S. enteria* also produces salmochelin by incorporating glucose residues on the 5' of two catecholamide rings of enterobactin to prevent siderocalin chelation of its siderophore (166,170,171).

VII. Zinc in the Human Body

The important role of iron for various cellular processes, including energy metabolism and participating in redox chemistry is well documented an understood (123). Other metals like magnesium (Mg^{2+}) or calcium (Ca^{2+}) are known for their roles in enzymatic processes and cellular signaling (172-174). Zn is another crucially important metal that is capable of participating in intra- and extracellular signaling and is a catalytic or structural component for over 3000 human metalloproteins and enzymes (175,176). Zn is distributed among various locations and organelles in eukaryotic cells with 30-40% of intracellular Zn localizing in the nucleus, 50% in the cytoplasm and the remaining 10% can be found within the membrane (177). In the extracellular environment, Zn can signal in an endocrine, autocrine, and paracrine fashion. Zn that is released into the presynaptic cleft can modulate the postsynaptic transmission and effect neuronal plasticity (178). Co-release of Zn and insulin from pancreatic β-cells can inhibit hepatic insulin clearance by inhibiting clathrin-dependent insulin endocytosis (179). Intracellular Zn acts as a second messenger after it is imported into the cytoplasm or released from organelle storage (180). Intracellular Zn signaling has been defined into two classes depending on their timescale, fast or "early" Zn signaling and late Zn signaling. Early Zn signaling occurs within seconds to minutes and does not require proteins or transcription factors for their signal transduction (180,181). Late Zn signaling involves the control of intracellular Zn concentrations

through the synthesis of Zn transport proteins and occurs hours after stimulation (175). Despite the intracellular Zn concentration being within the range of hundreds of micromolar, the "free" Zn concentration has been calculated to be in the picomolar range (182). The difference between total and available (free) Zn concentrations in cells has provided a new perspective on the role of Zn in cell regulation, cell signaling, and protein interactions (183). The proteins that use Zn as a catalytic or structural component must have high affinities for Zn in the range of the free Zn concentrations of the cell. Thus, low-affinity Zn regulatory sites may not be biologically relevant to cellular regulation and signaling (184). As with many other transition metals, the maintenance of Zn homeostasis is critical to mitigating the toxic effects that high concentrations can have, while ensuring enough Zn is present for cellular functions (175).

There are 24 membrane transporters of Zn encoded within the human genome. Out of the 24 transporters, 14 of them are Zrt, Irt-like proteins (ZIPs) and are responsible for Zn import into the cytoplasm from the extracellular space and cellular organelles (185). The 10 remaining membrane transporters are the Zn exporters (ZnT), which maintain cytosolic Zn homeostasis by mobilizing Zn from the cytosol into extracellular compartments or the lumen of cellular organelles (186,187). The mechanism of ZIP transport has yet to be elucidated; however, the ZnT transporters belong to the cation diffuser facilitator (CDF) superfamily and uses proton antiport to drive the transport of Zn into the extracellular and luminal space (188). In addition to these transporters, metallothionein also aids in regulating cellular Zn homeostasis.

A. Metallothioneins

Metallothioneins are small, low-molecular-weight, cysteine-rich, intracellular, Zn regulated proteins that can coordinate up to 7 atoms of Zn or other divalent cations like copper (Cu) or cadmium (Cd) (189). Between 5-15% of cytosolic Zn is bound by metallothioneins and

they are thought to contribute to Zn signaling by quickly adding or removing free Zn from the cytosol (190). Interestingly, unlike for iron storage, there is no dedicated high-capacity Zn storage protein similar to ferritin but the shuttling of Zn into endocytic vesicles may be an explanation for the lack of such a protein (175).

B.) S100 Proteins

S100 proteins are a family of small (between 10-12 kDa) acidic Ca^{2+} binding and signaling proteins that contain 2 EF-hand motifs (191,192). Unlike other calcium signaling proteins, S100 proteins have been shown to act both as intracellular regulators and extracellular signalers. Most S100 proteins are expressed in a cell-specific manner. Additionally, S100 proteins form obligate homo or heterodimers with the potential to form higher-order oligomers, which adds to their functional diversification (191,193). Extracellular S100 proteins are unique in their ability to act in a cytokine-like manner and interact with receptors for advanced glycation end-products (RAGE) and result in NF-κB gene transcription. Other than Ca^{2+} binding, a subset of S100 proteins have been reported to bind to $Zn^{2+,}$ and Cu^{2+} and this binding contributes to host nutritional immunity (194).

There are three S100 proteins that have been implicated for their role in nutritional immunity by virtue of their ability to sequester various transition metals from inflamed tissue (194). S100A7, also known as psoriasin due to its high concentrations in psoriatic plaques, has been recently shown have antimicrobial function against *E. coli* and *Pseudomonas aeruginosa* (195). The antimicrobial function of S100A7 has been attributed to Zn limitation and Zn complementation with excess Zn^{2+} abrogates S100A7 bactericidal activity (195). However, a second mechanism of antimicrobial activity has been proposed that relies on direct adherence to pathogens found on the epidermis to mediate antimicrobial activity (196).

S100A12 has been difficult to study due to its absence in the genome of mice (197). However human S100A12 has been found to be antimicrobial against several parasites (198). The mechanism of S100A12 antimicrobial activity has not been fully identified but studies into the antimicrobial properties of calcitermin, a protein homologous to the C-terminus of S100A12, supports the hypothesis of metal sequestration as the mechanism (199). Aside from Zn sequestration, S100A12 has also been suggested to bind to Copper (Cu^{2+}), which results in the production of superoxide that contribute to S100A12 antimicrobial properties (198,200).

The last and most well characterized S100 protein with antimicrobial function is calprotectin (CP). CP is unique among S100 proteins for the preference of S100A8 and S100A9 to heterodimerize, unlike most S100 proteins which are homodimers (201). The unique and characteristic heterodimerization between S100A8 and S100A9 gives CP the ability to sequester a wider range of transition metals such as Zn, Mn, Cu, and Fe and makes CP antimicrobial against a broad spectrum of pathogens (202-205). The binding of Ca^{2+} to CP allows for the high affinity transition metal sequestration, and therefore the concentration of Ca^{2+} modulates the function of CP (206). When CP is intracellular, concentrations of Zn and Mn are low enough that even during Ca^{2+} signaling cascades, CP does not meaningfully contribute to Zn or Mn sequestration. However, the higher levels of Ca^{2+} in the extracellular space ensures the activation of the metal-sequestering antimicrobial properties of CP in the location where it is most likely to encounter pathogens (206).

VIII. Metal-Acquisition Systems of *N. gonorrhoeae*

N. gonorrhoeae has evolved as a human specific-pathogen and is exquisitely adapted for survival within the human body. As such, it has developed an interesting strategy that allows it to acquire metals, like iron and zinc, during infection. *N. gonorrhoeae* utilizes several TonB-

dependent transporters that are highly specific for their human ligands to acquire metals during infection. TdTs are found as either single-component or two-component systems and hijack host proteins to remove the metals that are bound to them.

A. Two-Component TonB-dependent Transport Systems

Several TdT systems of *N. gonorrhoeae* consist of two partners with the first being the TdT and the second a lipoprotein (**Figure 2**). Typical TdT structure consists of a 22-stranded amphipathic β-barrel, 11 flexible loops extracellular loops, and folded plug domain inside of the barrel, which is located in the outer membrane of the bacteria (207,208). The associated lipoprotein also located tethered to the outer leaflet of the outer membrane. The transferrin- iron acquisition system, responsible for binding to hTf and stripping it of its Fe^{3+}, consists of the proteins TbpA and TbpB (209,210). TbpA is a typical TdT and structurally contains a 22-stranded β-barrel, 11 flexible extracellular loops, and a folded plug domain located inside of the barrel. TbpA/TbpB are encoded in the genome of most *Neisseria* and found in the genome of all isolates of *N. gonorrhoeae* and *N. meningitidis* (211). The necessity of the transferrin acquisition system was demonstrated in a human infection experiment where a strain created to be unable to produce TbpA/TbpB was attenuated for its ability to colonize the male urethra (212). The lipoprotein TbpB is bilobed with the N- lobe preferentially binding to hTf. *tbpA* and *tbpB* are organized within an iron-regulated bicistronic operon, where *tbpB* precedes *tbpA* (213). TbpA and TbpB are both capable of binding to human transferrin with nanomolar affinity, but only TbpA is required for the transport of iron across the outer membrane (214). TbpB has been hypothesized to make the iron acquisition more efficient since TbpB is only capable of binding to holo-transferrin and its absence results in a lower rate of iron uptake by the gonococcus (215).

The hLf iron acquisition system of the gonococcus is similar in nomenclature and structure to the transferrin acquisition system. The hLf acquisition system is comprised of a TdT termed LbpA and its cognate lipoprotein, LbpB (216,217). *lbpA* and *lbpB*, similar to the hTf system, are organized in an iron-repressed bicistronic operon where *lbpB* precedes *lbpA*.

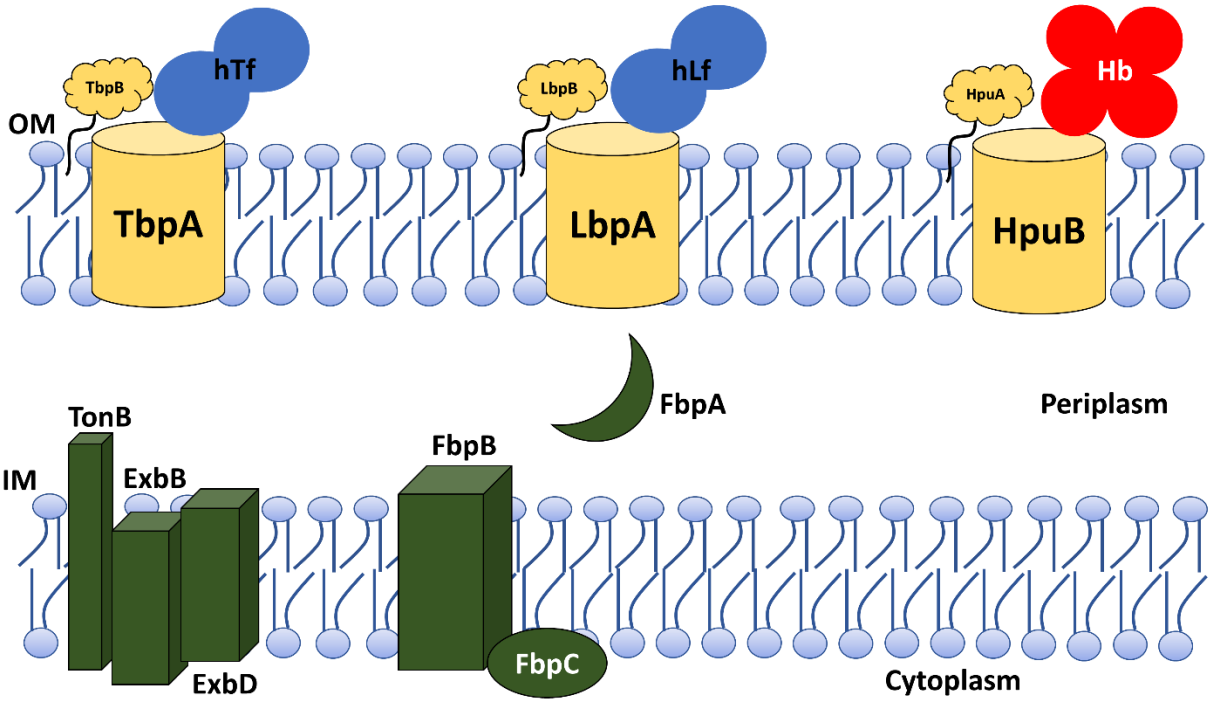

Figure 1.2. Two-Component TonB-dependent Transporters of *N. gonorrhoeae*

Figure 1.2. Two-Component TonB-dependent Transporters of *N. gonorrhoeae*.

Overview of the two component TonB-dependent transport systems: TbpAB, LbpAB, and HpuAB. The barrels imbedded in the outer membrane (OM) represent the TonB-dependent transporters (TdTs): TbpA, LbpA, and HpuB. Associated with each of these barrels are the lipoproteins: TbpB, LbpB, and HpuA. These are attached, through a lipid anchor, to the outer leaflet of the OM. The ligand for each of the TdTs is shown, with The TbpAB system bound to human Transferrin (hTf), the LbpAB system bound to human lactoferrin (hLf) and the HpuAB system bound to hemoglobin (Hb). Iron that is removed from hTf, hLf, and Hb traverses into the periplasmic space where it is bound by the periplasmic binding proteins FbpA. FbpA shuttles the iron atom to the ABC transporter FbpB and FbpC embedded on the inner membrane (IM), which uses cellular ATP to transport the iron atom into the cytoplasm of the gonococcus. The TonB complex of proteins, which consists of TonB, ExbB, and ExbD, are associated with the IM. TonB faces into the periplasmic space and interacts with the TonB box found in the plug domain of each of the TdTs. ExbB and ExbD harness the proton motive force that energizes TonB. Unlike the hTf system, the hLf system is subject to phase variation due to the presence of a polyC tract in the coding region of *lbpB* (216). The hLf system is present in all meningococcal strains, but in some 50% of gonococcal strains there is a deletion of *lbpB* and a 5' portion of *lbpA*. The hLf acquisition system is not essential for gonococcal virulence but provides redundancy for iron acquisition from the host (218).

The last of the two-component metal acquisition systems of *N. gonorrhoeae* is the hemoglobin/ hemoglobin-haptoglobin (Hg-Hp) iron acquisition system. The Hg system gene nomenclature is reversed from the hTf and hLf systems with the TdT being encoded by *hpuB* and the cognate lipoprotein being encoded by *hpuA* and is found in most *Neisseria*. The Hg system has been reported to bind to Hg and Hg-Hp complexes and requires both HpuA and HpuB for heme uptake and the ability to grow on hemoglobin as the sole iron source (219,220). The Hg system, similar to the hLf system, is subject to phase variation by a polyC tract in the coding region of *hpuA*.

B. Single-Component TonB-dependent Transport Systems

In addition to the two-component systems, *Neisseria* also encodes single component systems that lack the lipoprotein of the other transporters (**Figure 3**). The HmbR Hb acquisition system is a single-component acquisition system that allows *N. meningitidis* to acquire heme. The HmbR system is subject to phase variation via a polyG tract similar to the Hpu system (221,222). The *hmbR* gene exists as a pseudogene in *N. gonorrhoeae* due to the presence of a stop codon in its coding sequence (223). The iron-regulated xenosiderophore transporter FetA is another single-component system of *Neisseria*. FetA is a 76 kDa siderophore receptor, which is encoded within all *Neisseria* species (211,224). *Neisseria* species do not produce siderophores themselves but FetA allows for the gonococcus to use other bacterial siderophores during infection (224). FetA like most of the outer membrane proteins of *N. gonorrhoeae*, is subject to phase variation, which influences the expression level of FetA and depends on the number of cytosine residues between the -35 and -10 sequences (225). The gonococcus encodes 4 other single-component systems TonB dependent factor J (TdfJ), TonB dependent factor H (TdfH), TonB-dependent factor F (TdfF) and TonB-dependent factor G (TdfG).

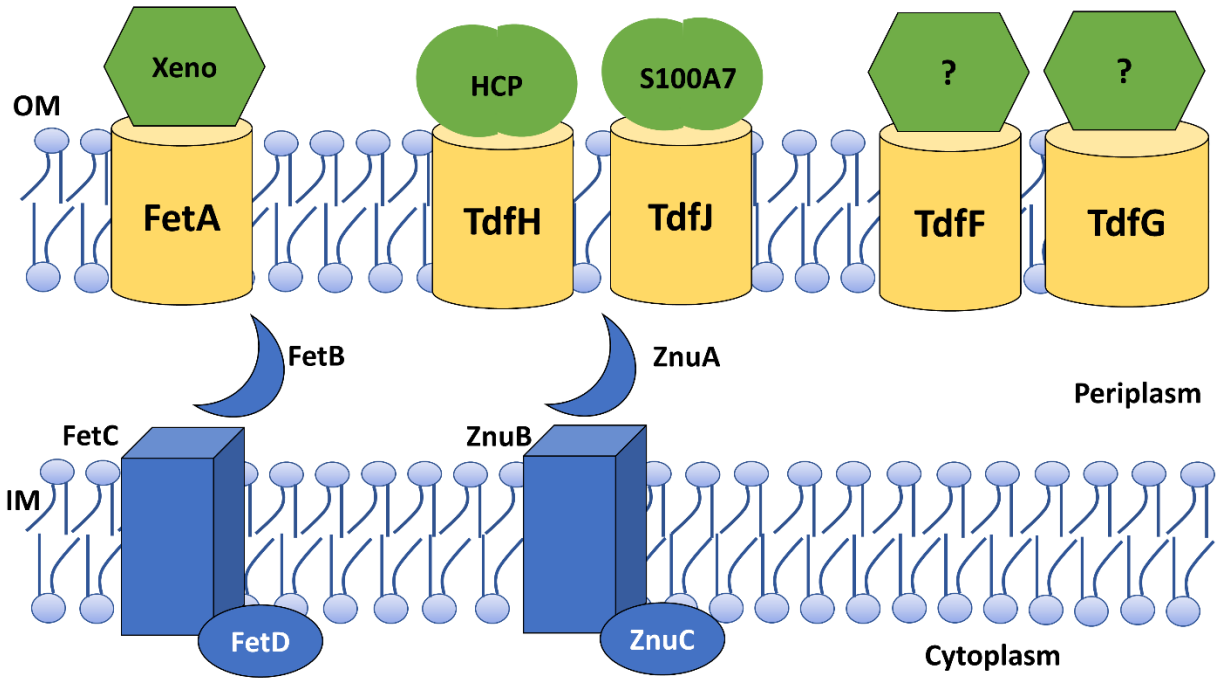

Figure 1.3. Single Component TonB-dependent Transporter of *N. gonorrhoeae*

Figure 1.3. Single Component TonB-dependent Transporters of N. gonorrhoeae. Overview of the Single component transporters, FetA, TdfH, TdfJ, TdfF, and TdfG of *N. gonorrhoeae*. The TonB-dependent transporters (TdTs) are barrels traversing the OM with the associated ligand for each system bound to the top of the barrel. The metal removal from the ligand results in the periplasmic binding protein sequestering the incoming metal and subsequently transports the metal to the appropriate ABC transporter. FetA internalizes xenosiderophores which are bound by the periplasmic binding protein FetB. FetB carries the xenosiderophore to FetC/FetD for the active transport of the xenosiderophore into the cytoplasm of the gonococcus. TdfH and TdfJ bind human calprotectin (hCP) and S100A7, respectively. The zinc associated with these proteins is removed and then shuttled into the periplasm where the periplasmic binding protein ZnuA binds the zinc atom. ZnuA carries the zinc ion to ZnuB/ ZnuC for the active transport of Zn into the cytoplasm of the gonococcus. To date neither TdfF nor TdfG have had their ligand identified, and it is unknown what ABC transport system they are associated with. TdfF and TdfG are both believed to be involved in iron homeostasis, and TdfG is the largest of the known gonococcal TdTs. TonB/ExbB/ ExbD is not depicted; however, they are required for FetA, TdfH, TdfJ, TdfH, and TdfG function.

Only TdfG remains uncharacterized, and is the largest of the TdTs encoded on the gonococcal genome at 130 kDa. While the ligand of TdfG remains unknown, it is iron-regulated and is primarily encoded in the genome of the gonococcus (226). The expression of TdfF is unique compared to the other TdTs of the gonococcus. TdfF was only expressed when gonococci were grown in cell culture medium supplemented with fetal bovine serum (FBS) and only found among the pathogenic *Neisseria*. Interestingly, TdfF was the only TdT that was important for the intracellular survival of the gonococcus during experimental infection of cervical epithelial cells and *tdfF* expression was upregulated in the absence of iron (227). The remaining two single component transport systems are unique for their role in zinc acquisition.

Initial studies into the function of the TdTs found that TdfH was not responsive to cellular iron levels, but TdfJ expression was enhanced in the presence of iron. This evidence suggested that the roles of these TdTs were for something other than iron transport (228). Gonococcal TdfJ and its homolog in *N. meningitidis,* ZnuD, are regulated via the zinc uptake regulator (Zur) (229). Recently, a detailed study of TdfJ elucidated that its interacting partner is the innate immunity protein S100A7 and its expression was enhanced in the presence of iron under Zn deplete conditions. Like almost all interactions between gonococcal TdTs, the interaction between TdfJ and S100A7 is human restricted, and mouse S100A7 was unable to support the growth of the gonococcus as the sole zinc source (230). TdfJ is regarded as a potential vaccine target due to the lack of known phase variation and its conservation within *Neisseria* (229).

The final single-component system of *N. gonorrhoeae* is the calprotectin binding and Zn-uptake system, TdfH, or CbpA for the meningococcal homolog. Similar to TdfJ, TdfH is subject to Zur regulation and repressed in the presence of Zn. The heterodimer of S100A8 and S100A9,

known as calprotectin, which is the ligand for TdfH, is abundant in inflamed tissue and has two high-affinity binding sites for Zn (202,205). The interaction between TdfH and calprotectin allows for gonococcal survival of neutrophil extracellular traps (231).

C. TonB-Dependent Transport

The transport systems described above are all members of the TonB dependent family of outer-membrane receptors. The transport of the heme, iron chelates, Fe, and Zn through the β-barrel of these transporters requires energy that is mobilized through the TonB-complex (232,233). The TonB complex is a tripartite system of proteins consisting of TonB, ExbB, and ExbD located in the periplasmic membrane. TonB, ExbB, and ExbD are encoded on the gonococcal genome in an iron-repressed operon (234). TonB has 3 functional domains: an N terminal transmembrane domain, a proline-rich spacer that allows for the protein to extend into the periplasmic space, and a C-terminus that interacts with the TonB box present on the plug domain of the TonB receptor (207,235). TonB harnesses the energy from a proton motive force (PMF) and through the interaction of TonB with the TonB box, the energy from the PMF enables the acquisition of metal substrates through a yet undetermined mechanism (236).

D. Cytoplasmic transport

After the import of the metal nutrients through the TonB outer-membrane receptors, the transport of these nutrients to the cytoplasm is facilitated through a set of periplasmic binding proteins (PBP), an inner membrane permease and ATP-binding cassette (237). Iron transport in the periplasm from hTf and hLf is accomplished via the ABC transport system of FbpABC. FbpA is the PBP and when unbound by Fe^{3+}, is hypothesized to interact with TbpA and drive Fe transport through TbpA due to its affinity for Fe^{3+} (~10^{-18} M) (238,239). There is a separate ABC

transport system FetCDEF, which allows for the utilization of enterobactin and requires the PBP FetB (240,241). No dedicated heme transport system has been discovered in *Neisseria* but the presence of a heme ABC transport system is hypothesized to exist. The ZnuABC transport system allows for the import of Zn and is Zur regulated in *N. meningitidis* (242). ZnuA is the PBP and is required for the growth of the gonococcus on S100A7 and Calprotectin (230).

E. Regulation

The ability of an organism to sense their metal environment and respond during periods of high and low concentrations is critical for their survival and the virulence for pathogens. The iron acquisition systems of *N. gonorrhoeae* and their regulation is crucial for maintaining enough iron to grow and survive, but not allow for toxic accumulations (243). Thus, regulation of the iron acquisition systems is driven by the ferric uptake regulator (Fur) and is a part of a global iron regulatory network called the Fur regulon (244). When cellular concentrations of iron are high, monomeric Fur interacts with ferric iron to make dimeric Fur. The dimer is the functionally active form and binds to Fur boxes on the DNA of genes in the Fur regulon to regulate transcription (245). Traditionally this regulation was the repression of genes when iron stores inside of the cell were high. As the intracellular stores of iron were used, the repression was relieved due to a lack of iron capable of dimerizing Fur (246). Studies with *N. meningitidis* and *N. gonorrhoeae* have seen independent FetA activation via the Fur repressed AraC-like regulator MpeR (241,247,248). The Zn transport systems, TdfH and TdfJ, are regulated similar to the iron systems but belong to the Zur regulon and are under Zur regulation. Similar to Fur, when the intracellular stores of Zn are high Zur monomers bind to Zn^{2+} and form dimers (249). These dimers bind to Zur boxes on the DNA of Zur regulated genes and in the case of TdfH and TdfJ, repress their transcription (242,250).

IX. Animal Models of Infection

N. gonorrhoeae is an obligate human pathogen and is highly adapted for survival inside of a human host. This host adaptation has made it so that no animal models accurately mimic human infection. This inadequacy is due to the number of host restricted proteins essential for effective colonization and infection (251-253). Experimental genital tract infection has only been successful in chimpanzees and estradiol-treated mice. The chimpanzee model of genital tract infection is no longer available making the mouse model the only viable animal model for evaluation of genital tract infections caused by *N. gonorrhoeae* (254,255). Treatment of mice with 17β-Estradiol prevents overgrowth of commensal bacteria and extends the proestrus phase of the mouse estrus cycle which is the optimal colonization phase (256,257). Recently, a human transferrin transgenic mouse was generated and has been tested as an infection model with *N. meningitidis.* While this model has shown promise, more studies are needed to determine its ability to mimic human infection with both the gonococcus and meningococcus (258). Additional insertions of human receptors critical to infections like carcinoembryonic antigen cellular adhesion molecules (CEACAM), or Factor H may alleviate some of the difficulties associated with animal models of this obligate human pathogen.

X. Vaccination efforts

The continued rise in gonococcal infections, the dwindling number of clinically useful antibiotics, and the lack of a protective immune response after infection highlights the need the need for a new therapeutic gonococcal vaccine. These efforts have been hampered by the antigenic variability, phase variation exhibited by many surface-exposed proteins of the gonococcus, and the active suppression of an adaptive immune response (56,57). Further complicating vaccine efforts is the ability of the gonococcus to prevent the deposition of

antibody on its surface. Gonococcal LOS that is decorated with sialic acid has been shown to reduce the deposition of antibodies to porin proteins (259). The production of a conserved protein RmpM acts as a decoy protein and prevents the deposition of bactericidal antibodies onto gonococcal porin (260,261). An effective gonococcal vaccine will need to induce an immune response within the mucosal tissue of the genital tract, which is devoid of organized lymphoid tissue (262,263). New strategies at inducing mucosal immune responses through intranasal vaccinations have shown promise at generating an immune response in the genital tract (264,265).

The selection of an effective antigen is crucial for the development of an effective gonococcal vaccine. Attempts to generate a pilin-based vaccine were unsuccessful even though there was an abundant production of antibodies (266). Conserved portions of pilin are immunosilent and thus, the immune response is driven by the highly variable portions of pilin, which offer no cross-protection for strains expressing a different pilus (267). Since this attempt, no other human trials of a gonococcal vaccine have been conducted; however, a recent study found intravaginal vaccination with gonococcal OMVs administered with microencapsulated IL-2 as adjuvant resulted in protection from gonococcal infection. This immunization method resulted in cross-protection across numerous strains of *N. gonorrhoeae* and provided clear evidence that with the correct immunogen, a protective immune response can be achieved (268,269). A newly enticing vaccine antigen is the heptose-linked 2C7 epitope present within gonococcal LOS (270). Despite the phase-variable nature of LOS, there is a strong selection for the presence of this epitope during experimental infection. Passive delivery of 2C7 monoclonal antibody protects against infection in mice and hexamerized 2C7 monoclonal antibody was

bactericidal (271). A gonococcal vaccine using a peptide mimic of this epitope is currently under development (272).

The conservation of TdTs and their limited antigenic variability has also added them to the potential list of viable immunogens that could protect against gonococcal infection. The use of TdTs is thought to provide the additional benefit of starving the gonococci of the essential metal nutrients while also inducing cross-protective and bactericidal antibodies. Early studies investigating the efficacy of the hTf system as an immunogen found that when a TbpA-Ctb conjugate vaccine was administered intranasally to mice, there was an induction of cross-protective antibodies. A TbpB-Ctb conjugate vaccine was more immunogenic but its antibodies were limited in their cross-protection (264). This discovery has led to the generation of new "hybrid" antigens that allow extracellular-loop epitopes of TbpA to be presented in the context of a TbpB scaffold. This is hypothesized to allow for maximal antibody induction that is seen with TbpB based immunizations while maintaining the cross-reactive antibody production induced by the TbpA based immunizations. Immunization with hybrid antigens of TbpA/TbpB in mice resulted in the production of bactericidal antibodies and provided modest protection against lower genital tract infection of *N. gonorrhoeae* (273). These studies highlight the promise of TdTs as credible antigens for the protection against gonococcal disease and further studies investigating other TdTs potential for protection are needed.

Chapter 2: Methods and Materials

I. Bacterial growth and maintenance

Escherichia. coli strains used in this study were routinely cultured in Lauria Bertani broth (274) supplemented with appropriate selection as follows: ampicillin at 100 μg/mL, chloramphenicol at 34 μg/mL, and kanamycin at 50 μg/mL. Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 1 mM was used for the induction of complementation plasmids, which allows for *macA* gene transcription under the control of the lac promoter. Gonococcal strains were maintained on gonococcal base media (GCB, Difco) supplemented with Kellogg's supplementation (275) and 12 μM $Fe(NO_3)_3$. Iron stress was achieved by passage of isolated gonococcal colonies onto GCB media supplemented with 5 μM deferoxamine (Desferal, Sigma Aldrich). Lyophilized desferal was resuspended in sterile deionized water at a stock concentration of 50 mM. For liquid culture and growth curve analysis, gonococcal isolates from GCB-desferal plates were used to inoculate chemically-defined chelex-treated media (CDM)(276). To achieve Zinc (Zn) restriction, cells previously grown on GCB media supplemented with 12 μM $Fe(NO_3)_3$ were inoculated into CDM and incubated at 37°C with 5% CO_2 with shaking at 225 RPM for one mass doubling event, approximately 1-2 hour(s), before addition of N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamine (TPEN, Sigma Aldrich) to a final concentration of 1 μM. TPEN stocks were generated by diluting TPEN in 100% ethanol at a stock concentration of 1mM. Cultures were incubated at 37°C with 5% CO_2 and shaking at 225 RPM for 3 hours before being standardized to 100,000 Klett unit μL for whole-cell lysates, or 15,000 Klett unit μL for blotting onto nitrocellulose membranes.

II. Small Molecule Inhibitors ELISA

Single colonies of gonococci grown on GCB containing 50 μM Desferal were swabbed with a cotton-tipped swab and suspended in Phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM N_2HPO_4, 176.35 μM K_2PO_4) to a final OD_{600} of 1.0. The resuspended culture was added to a 96-well plate, coated in 0.01% poly-L-lysine overnight, and incubated at room temperature for 1 hour. Gonococcal cultures were removed from the plate before a 1% casein blocker in PBS blocker (ThermoFischer) was added. The plate incubated at room temperature for one hour before the addition of small molecule inhibitors in the 1% casein blocker at a final concentration of 100 μM was added to the wells. Unlabeled human transferrin at a concentration of 2mg/mL (hTf, Sigma Aldrich) was used as a competitive inhibitor as a positive control of hTf inhibition. The inhibitors were removed from the plate and 12 nM hTf conjugated to horseradish peroxidase (hTf-HRP) was added to the wells. The plate was incubated with hTf-HRP for one hour before being washed five times with PBS. The plate was developed with 3,3',5,5'-tetramethylbenzidine (TMB, ThermoFischer) for 5-15 mins. 3 M sulfuric acid was added to stop development before the plate was read on a Vmax microtiter plate reader at 420 nm.

III. Testing Tf binding by TbpA mutants via ELISA

Iron-stressed gonococci grown overnight on GCB with 50 μM desferal was swabbed and suspended into PBS at 1.0 O.D.600. Cultures were added to a Maxisorb 96-well plate (ThermoFischer) coated in 0.01% poly-L-lysine and incubated at room temperature for 1 hour. Cells were removed from the plate, and 3% bovine serum albumin (BSA) in PBS was added to the wells as a blocker. Wells were washed three times with PBS before the addition of 12 nM

HRP- hTf. Plates were incubated for 1 hour at room temp before being washed five times with PBS. Plates were developed with TMB before 3M sulfuric acid was added to stop development. Plates were read in a microtiter plate reader at 420nm.

IV. Iron Saturation of hTf and bTf and Growth Premix Generation

Apo-bovine (bTf) and -human transferrin (hTf) was dissolved in initial buffer (100 mM tris, 150 mM NaCl, 20 mM $NaHCO_3$) at 10 mg/mL. Ferration solution (100 mM sodium citrate, 100 mM $NaHCO_3$, 5 mM FeCl3- 6H20) was added to bTf or hTf in two-fold molar excess to achieve desired saturation percentage. The proteins and ferration solution nutated at room temperature for one hour. Solutions were added to pre-wet 10,000 Da cutoff Slide-A-Lyzer dialysis cassettes (ThermoFisher) and dialyzed against dialysis buffer (40 mM Tris, 150 mM NaCl, 20 mM $NaHCO_3$) for 2 hours at room temperature before the buffer was swapped for fresh buffer and dialyzed overnight at 4^{o}C. Wild-type human calprotectin (WT-CP), site 1 knockout (S1KO) human calprotectin, site 2 knockout human calprotectin (S2KO), double site knockout human calprotectin (DKO), and murine calprotectin (MCP), supplied by Dr. Walter Chazin, was diluted with PBS to 100 μM before being saturated with ZnSO4 at a 2:1 ratio of CP dimer to Zn except for the S1KO and S2KO, which were mixed with a 4:1 ratio of CP dimer to Zn in order to maintain equivalent levels of protein saturation. In order to restrict the growth of the gonococcus to the desired iron and zinc sources, concentrated growth premixes were made and contained a final concentration after dilution with culture of hTf at 7.5 μM, bTf at 2.5 μM, 10 μM HCP or MCP and 5 μM TPEN. A 5 μM ZnSO4 free Zn premix that did not contain TPEN was used as a positive control and a Zn devoid premix containing everything but a Zn source was used as a negative control for Zn dependent gonococcal growth.

V. Generation of TbpA Double Mutants

E. coli containing the plasmids pVCU190 or pVCU191, which were originally generated by Dr. Devin Cash, were grown overnight in LB with the selection of 34 μg/mL chloramphenicol. Plasmids were prepped via Qiagen miniprep kit and subjected to SacI (New England Biolabs) restriction endonuclease digestion overnight at 37ºC. Linearized plasmid was precipitated in 100% ethanol and 120 mM sodium acetate overnight at -20ºC. Digested plasmids were removed from the freezer and centrifuged at max speed for 10 minutes, after which, the DNA pellet was washed with 70% ethanol and subjected to another centrifugation at max speed for 10 minutes. The DNA pellet was air-dried before being resuspended in sterile DNase- RNase free deionized H_2O. Piliated FA19 was streaked from freezer stock onto GCB plates and incubated as described above. Piliated colonies were passed onto GCB plates and incubated overnight before being suspended in 1 mL of GCB Alternative media (GCB containing 50 μM $MgCl_2$, 130 μM $CaCl_2$, 17.76 mM Glucose, 547.2 mM L-glutamine, 0.376uM thiamine pyrophosphate). In a separate tube GCB alternative media, 10 μL of piliated FA19 cell suspension and 1 μg of linearized plasmid DNA were mixed gently and incubated for 30 minutes at 37ºC with 5% CO_2 without shaking. Fresh GCB alternative media was added to the wells of a 6-well cluster dish, and the 105 μLs of cell suspension was added to the cluster dish to a final volume of 900 μL. The plate was incubated for 5 hours at 37ºC with 5% CO_2 before plating. The transformation reaction was plated in 10 or 100 μL aliquots onto GCB media containing 1 μg/mL chloramphenicol and incubated for two days at 37ºC with 5% CO2. After two days, colonies were single colony purified via serial passage onto GCB media plates supplemented with chloramphenicol at 1 ug/mL. A colony PCR for *tbpA* using primers oVCU 747 and oVCU748 was performed and the

PCR produced was sent for sequencing to confirm the presence of the mutations in the gonococcal chromosome. A PCR of *tbpB* using primers oVCU 750 and oVCU752 was visualized using a 1.5% agarose ethidium bromide gel to confirm the presence or deletion of *tbpB*.

VI. *macA* deficient strain growth assays for gonococci heme sensitivity

FA19, *macA::kan, pGCC3+::macA^C, pGCC4+::macA^C* gonococcal strains were iron starved overnight as previously described, and single colonies were used to inoculate CDM that contained 1 mM IPTG. Strains were initially grown for approximately 2 hours or until one mass doubling event. Cultures were standardized to 0.02 OD_{600} and plated in a 96-well microtiter plate. Each well contained 2.5 μM apo-bovine transferrin as a chelator, and either 7.5 μM hTf or heme as the sole iron source available to the gonococcus. Final heme concentrations of 25 μM, 15 μM, 10 μM and 5 μM were used as iron sources. Plates were incubated at 37ºC with 5% CO2, with shaking at 225 RPM, for 6 hours with hourly OD_{600} readings.

VII. Isothermal Titration Calorimetry

Recombinant TdfH purified from E. coli inclusion bodies at a final concentration of 20 μM in a PBS+ 0.05% n-Dodecyl-B-D-Maltoside (PBS-DDM) buffer was generously provided by Dr. Nicholas Noinaj for the following isothermal calorimetry experiments. WT-Cp, S1KO, (ΔHis6), S2KO (ΔHis3, Asp), and DKO (ΔHis6- ΔHis3, Asp) provided by Dr. Walter Chazin, was buffer exchanged into PBS-DDM buffer via size exclusion chromatography. Relevant fractions were pooled and concentrated to 200 μM. The reference cell and sample cell of the NanoITC instrument were washed five times with PBS-DDM, before the addition of 300 μL of 20 μM TdfH. Increasing concentrations of CP were incrementally titrated into TdfH over twenty

injections and the changes in temperature were recorded. Analysis and fitting were performed via origin through a multi-site fit.

VIII. CP dependent Growth Assays

FA19 was streaked from freezer stocks and patched onto full GCB plates before inoculation into CDM. Cells were used to inoculate a trace-metal free sidearm flask containing CDM to a density of 20 Klett units (KU). The cells were incubated at 37°C with 5% CO_2 and 225 RPM until a Klett of 40 KU was reached (approximately 1 hour) after which, the culture was back-diluted to 0.02 OD_{600} with fresh CDM. 100 μL of the back-diluted culture was added to a 96-well plate that was previously loaded with the various concentrated growth premixes. The plate was incubated for 12 hours at 37°C with 5% CO_2 and orbital shaking in a BioTek Cytation5 plate reader with absorbance readings taken every hour. Data were analyzed in GraphPad Prism using a two-way ANOVA with *post-hoc* comparisons performed with Tukey's multiple comparisons test.

IX. Whole-Cell Dot Blot Competition Assays

Gonococcal strains FA1090 (WT), MCV 661 (*tdfH::*Ω), MCV 662 (*tdfJ::*Ω) and MCV 936 (*tdfJ::*Ω, *tdfH::Kan*) were passaged on GCB media plates (Difco) two days before Zn restricted liquid growth. Colonies from GCB plates were used to inoculate trace metal-free flasks, and cultures were incubated at 37°C with 5% CO_2 with shaking at 225 RPM for around 2 hours before the addition of a final concentration of 1 μM TPEN. Cultures incubated for approximately 4 hours at 37°C with 5% CO_2 before blotting onto a nitrocellulose membrane at a standardized culture density of 30,000 Klett unit μL. Blots containing gonococci were dried overnight before blocking with 5% skim milk in low salt tris buffered saline (LS-TBS, 50 mM Tris, 150 mM NaCl) for 1 hour. Blots were probed with 0.1 μM human calprotectin conjugated

to horseradish peroxidase (HCP-HRP), HCP-HRP mixed with five- or ten-fold molar excess of unlabeled HCP, or HCP-HRP combined with five- or ten-fold molar excess unlabeled MCP competitor for 1 hour. Blots were washed with LS-TBS before being developed with DAB metal substrate (Thermofischer) or Opti-4CN (Bio-Rad). The densitometries of scanned triplicated blots were analyzed using Bio-Rad Image Lab.

X. Total Calprotectin Binding Assay

Nitrocellulose membranes generated the same as the competition assay, were blocked with 5% Skim milk in LS-TBS for 1 hour before being incubated with either 0.5 μM HCP or 0.5 μM MCP for 1 hour. Blots were washed with LS-TBS + 0.1% TWEEN 20, three times for 10 mins each, before being probed with a 1:100 dilution of αS100A9 antibody (ThermoFisher) diluted in 5% skim milk blocker for 1 hour. Blots were washed, as before, in LS-TBS + 0.1% TWEEN-20 and then goat α-mouse IgG conjugated to HRP secondary antibody (BioRad) at a 1:3000 dilution in 5% skim milk blocker was added to the blots for 1 hour. A final washing step after secondary antibody incubation was performed, as the previous two, before development with DAB C/N (ThermoFisher). Blots developed for approximately 10-15 mins before being scanned for densitometry analysis.

XI. Alignment of human and mouse S100A8 and S100A9 protein sequences

The sequences of human S100A8 (Accession number: AAH05928.1), S100A9 (Accession number: AAH47681.1), mouse S100A8 (Accession number: NP_038678.1), and S100A9 (Accession number: NP_001268781.1) were pairwise aligned through Geneious using a Blossom 65 matrix. This alignment was then fed into ESPript 3.0 to produce the final alignment, which includes the secondary structural elements taken from PDB ID 4GGF. The residues different between human and mouse were then mapped to the surface of the human calprotectin structure

using PyMOL (Schrödinger) and final figures prepared and assembled in Adobe Photoshop and Illustrator.

Bacterial expression and purification of wild-type, mutant hCP, and mouse CP followed previously described protocols (204,277) and was supplied and performed by Dr. Walter Chazin from Vanderbilt University. The Zn binding site knock-out mutants (site 1- S1KO, site 2- S2KO, both sites- TKO) have His-Asn substitutions for the 4 conserved His residues in site 1 and His-Asn substitutions for the 3 conserved His residues plus an Asp-Ser substitution for the conserved Asp residue in site 2. Mouse S100A8 and S100A9 in pQE32 vectors, a kind gift from Professor Claus Heizmann, were reengineered to remove the His tags. The protein was purified following the protocol used for human CP. Briefly, plasmids were transformed in C41 *E. coli* cells following standard procedures. For each protein, when the OD_{600} reached 0.6, cells were induced at 37 °C by the addition of 1 mM IPTG and allowed to grow 4-12 hours post-induction. Cells were harvested by centrifugation (6.5 krpm, 20 minutes, 4 °C) and re-suspended in Lysis Buffer (50 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM PMSF, 0.5 % Triton X-100). Cells were then sonicated (10 min, 50 watts, 5 seconds on/10 seconds off) and centrifuged at 20,000 rpm for 20 min. The supernatant was discarded and the pellet was re-suspended in Lysis Buffer then sonicated and centrifuged as previously. The pellet was then re-suspended in 4 M guanidinium-HCl, 50 mM Tris pH 8.0, 100 mM NaCl, and 10 mM BME. The solution was centrifuged at 20,000 rpm for 20 min, then dialyzed against 20 mM Tris pH 8.0 and 10 mM BME. The dialysis buffer was changed 3 times over the course of 12 hours. The solution was centrifuged, filtered and loaded onto a SepharoseQ column (GE) (flow rate = 4 ml/min). After loading, the column was washed with 3 CV Buffer A (20 mM Tris pH 8.0, 10 mM BME) and eluted with a gradient (10 CV, 0→0.5 M) to Buffer B (20 mM Tris pH 8.0, 1 M NaCl, 10 mM

BME). Relevant fractions were pooled, concentrated, and loaded onto a S75 column. Protein was eluted with 1CV S75 Buffer (20 mM Tris pH 8.0, 100 mM NaCl, 10 mM BME). Relevant fractions were pooled, flash frozen, and stored at -80 °C.

Recombinant TdfH was supplied by Dr. Nicholas Noinaj from Purdue University. The TdfH-CP complex formation studies and TdfH homology modeling were also done at Purdue by the Noinaj Lab. Briefly, the full-length *tdfH* gene (*Neisseria gonorrhoeae*; NGO0952) was codon-optimized for expression in *E. coli* (Bio Basic). The *tdfH* gene was subcloned into the pHIS2 plasmid using NcoI and XhoI restriction sites. Expression into inclusion bodies was performed in BL21(DE3) cells, induced by addition of 0.2 mM isopropyl-D-1-thiogalactopyranoside (IPTG) after growth to an OD_{600} of ~1.0, and induced at 37ºC for 3 hours. The cells were then harvested and resuspended in 1x PBS pH 7.4 (10 mL per gram of cell paste) supplemented with phenylmethylsulfonyl fluoride (200 μM final concentration) and DNaseI (10 μg/mL final concentration). The cell suspension was lysed by three passes through an Emulsiflex C3 (Avestin) at 15,000 psi. The lysate was centrifuged at 7,000 x *g* for 20 min at 4 ºC, and the pellet washed three times with 1x PBS supplemented with 1% triton X-100 and 5 mM (Ethylenediaminetetraacetic acid) EDTA pH 7.4, one time with 3 M urea in 1x PBS, and two times with 1x PBS with 5 mM EDTA pH 7.4 using a dounce homogenizer.

Washed inclusion bodies were resuspended to 5-10 mg/ml in 8M urea containing 2.5 mM β-mercaptoethanol (BME) in a dounce homogenizer and supplemented with 0.5% sarkosyl. This was mixed for 15 min at room temperature and then centrifuged for 15 min at 32,000 x *g*. The supernatant was then diluted 60% in refolding buffer [20 mM Tris-HCl pH 8.0, 200 mM NaCl, 10% glycerol and 0.17% n-Dodecyl-B-D-Maltoside (DDM)] and dialyzed overnight at 4 ºC against a 20x volume of 1x PBS pH 7.4.

The dialyzed sample was centrifuged at 32,000 x *g* for 15 mins at 4 ºC and further purified using a linear gradient (25 - 300 mM imidazole) with a Ni-NTA column attached to an AKTA Purifier (GE Healthcare) in Buffer A (1x PBS pH 7.4 buffer, 0.05% DDM) and Buffer B (1x PBS pH 7.4 buffer, 0.05% DDM, 1 M imidazole). Peak fractions were verified by SDS-PAGE and the purest fractions were combined and treated with TEV protease at 4 ºC overnight in dialysis into 1x PBS. The sample was then passed over a second Ni-NTA column and the flow-through concentrated and further purified using a Superdex 200 Increase 10/300 GL column (GE Healthcare) in 1x PBS pH 7.4 supplemented with 0.05% DDM.

Table 1.1 Gonococcal Strains and Plasmids

Strain	Genotype	Reference
FA1090	WT *lbpBΔ*	(278)
FA19	WT	(279)
FA6905	FA19 *tbpBΔ*	(252)
FA6815	FA19 *tbpB::Ω*	(215)
MCV 661	FA1090 *tdfH::Kan*	(227)
MCV 662	FA1090 *tdfJ::Kan*	(280)
MCV 936	FA1090 *tdfJ::Ω, tdfH::Kan*	(231)
MCV 168	FA19 *tbpBΔ*, TbpA K359R	(281)
MCV 210	FA19 *tbpBΔ*, TbpA D251A	This Study
MCV 211	FA19 *tbpBΔ*, D251A/ K359R	This Study
Stellar Top10	F–, endA1, supE44, thi-1, recA1, relA1, gyrA96, phoA, Φ80d lacZΔ M15, Δ (lacZYA - argF) U169, Δ (mrr - hsdRMS - mcrBC), ΔmcrA, λ–	Takara Clonetech

Plasmids	Genotype	Reference
pVCU 190	pUNCH 755 TbpA D251A	This Study
pVCU 191	pUNCH 755 D251A/ K359R	This Study
pUNCH 1304	pCRII *hemH*::*CAT*	(282)

Chapter 3: Affinity and Species Specificity of the Interaction Between TdfH of *Neisseria gonorrhoeae* and Its Ligand, Human Calprotectin

Introduction

Neisseria gonorrhoeae is responsible for the sexually-transmitted infection gonorrhea and has resulted in a steady rise in infections worldwide over the last decade (283,284). In 2018 alone, the number of reported gonococcal infections reached over 500,000 in the United States (283). Increasing antimicrobial resistance among recently-isolated strains has complicated the treatment of this infection (60,285). The accumulation of antimicrobial resistance has left clinicians with few remaining therapies. The current CDC-recommended treatment is dual therapy with ceftriaxone plus azithromycin (60). A recent case study in the United Kingdom reported a patient infected by a gonococcal strain exhibiting high levels of resistance to both drugs in the dual therapy, marking the beginning of an era where there may be no effective treatments for gonococcal infections (61,286). The lack of protective immunity against *N. gonorrhoeae* after infections, coupled with the closing window of treatments available, highlights the need for new therapeutics or ideally vaccine interventions that would prevent gonococcal diseases.

Previously, four major attempts at generating a gonococcal vaccine have all failed their respective clinical trials likely due to the antigenic diversity of *N. gonorrhoeae* surface structures (287) . Whole cell, partial autolyzed cell, porin, and pilus based vaccines were unable to induce a cross protective immune response and were unable to protect individuals from future

gonococcal infections (266,288-290). The pilus based vaccine attempt was one of the first attempts at gonococcal vaccination and demonstrates the problems with generating a cross protective immune response (266). Gonococcal pilus genes undergo high frequency antigenic variation, which results in a new pilin variant in every 10^2-10^3 cells (70,71). The pilin-based vaccine was capable of stimulating a robust immune response and showed protection, but the protection included preventing infections from cells expressing only the exact same pilus protein (266). The number of pilin protein variants that can be generated effectively removed the use of pilin as a viable vaccine candidate, and is a common issue among many gonococcal surface proteins like those used in the whole cell, porin, and partially autolyzed vaccines. The sheer antigenic diversity of gonococcal surface proteins necessitates the need to search for more suitable vaccine candidates. It is our belief that the incorporation of conserved outer-membrane proteins which exhibit minimal antigenic variation or phase variation, and are immunogenic, will provide the best chance of success for a future protective-gonococcal vaccine.

In order to inhibit microbial invaders from multiplying, mammalian hosts deploy "nutritional immunity" as a means to restrict metals via the production of metal-binding proteins (129). This protective mechanism was first described in the context of iron deprivation but extended to other transition metals as well (129,291). Metal sequestration, as well as tight control of metal metabolism, leaves the human body depleted for free metals. *N. gonorrhoeae* is highly effective at subverting host nutritional immunity by hijacking human metal-binding proteins and using the metal cargo for growth and survival (129,131,209,292-294). This "metal piracy" is accomplished via a family of outer-membrane transporters, known as TonB-dependent transporters (TdTs). These transporters depend on the TonB-ExbB-ExbD complex of proteins to harness the energy generated by the proton motive force across the inner membrane (207,295).

The gonococcus can utilize iron-bound to human transferrin and lactoferrin as metal sources and has recently been shown to utilize S100A7 for Zn-dependent growth (209,230,296). TdTs are highly conserved, demonstrate limited antigenic variation and, in the case of TbpA, promote the generation of cross protective antibodies. Therefore, TdTs have been the subject of recent attempts at the development of a gonococcal vaccine (273).

The gonococcal genome encodes eight known TdTs, with five of these transporters, TbpA, LbpA, HpuB, TdfJ, and TdfH, binding to a known host ligand (226,295). Iron acquisition via transferrin is accomplished through TbpA, which demonstrates a species specificity for only human transferrin (252,296). Similarly, Zn acquisition through S100A7 is achieved via the production of the gonococcal transporter TdfJ and exhibits a similar species restriction for ligand binding (230). *N. gonorrhoeae* has also been shown to utilize human calprotectin (hCP, composed of a heterodimer of S100A8 and S100A9) for survival in neutrophil extracellular traps [NETs]) (231).

Calprotectin (CP) is one of its most abundant cytosolic proteins found within neutrophils and can also be found within the primary and secondary neutrophilic granules (297). When neutrophils undergo the process of NET formation, NETosis, CP is secreted into the extracellular environment and contributes to the antimicrobial activity of NETs through its metal sequestering properties (298-300). The metal sequestration exhibited by CP is attributed to its two metal-binding sites, which are found on opposite sides of the dimer interface. These sites are defined by the residues that are responsible for metal chelation. The first site, known as site 1, is composed of 6 histidine residues, which is different than the metal coordinating residues found within other S100 proteins (301). Site 1, and its unique makeup, allows for the flexibility of binding more than just Zn (301). The second site, known as site 2 has the canonical set of residues found

within other S100 proteins, three histidines and an aspartic acid residue. Unlike site 1, site 2 only has the ability to bind to Zn (205). Zn piracy from CP has been described as being TdfH-dependent in *N. gonorrhoeae* (231,302).

TdfH is highly conserved among the pathogenic *Neisseria* species making it a promising candidate for vaccine or drug design (302). In our studies here, as is true for other protein-protein interactions in the pathogenic *Neisseria*, the TdfH-CP interaction is demonstrated to be human restricted. Zn-loaded mouse calprotectin was not capable of supporting the growth of the gonococcus in a TdfH-dependent manner. Using isothermal calorimetry, the binding of TdfH with hCP fit a two-state model characterized by low and high-affinity binding with micromolar and nanomolar affinities, respectively. The growth deficiency of *N. gonorrhoeae* with the S1KO hCP point mutant, which is unable to bind Zn at the non-canonical metal-binding site, indicates that metal scavenging may occur optimally at Site 1. We find that TdfH is necessary and sufficient for hCP binding, resulting in Zn acquisition directly from hCP. Our studies provide molecular insight into the interaction of TdfH with hCP and allow us to form a working structural model for the TdfH/hCP complex in the piracy of Zn. This insight provides the blueprint for further investigations of the vaccine potential of TdfH and new therapeutics that disrupt the TdfH-hCP interaction.

I. Results

A. *N. gonorrhoeae* growth is not supported by mCP and preferentially binds hCP

Jean et al. (231) demonstrated that the gonococcus could use CP in a TdfH-dependent fashion, resulting in Zn accumulation. Furthermore, this study demonstrated an *in vivo* relevance for the production of TdfH in that production of this transporter enabled the gonococcus to better survive killing by neutrophil extracellular traps (NETs)(231). While Jean et al. demonstrated a direct interaction between CP and whole, TdfH-producing gonococcal cells, direct protein-

protein interactions were not previously shown, nor was the mechanism or affinity of the interactions measured. In the current study, we investigated whether the interactions between TdfH and CP were species-specific. Previously-studied gonococcal TdTs bind and acquire metals specifically from the human forms of their ligands (230,252,303). To test if this was also true for TdfH, FA19 cells grown in CDM were supplemented with ~25% saturated mCP as the sole Zn source according to previously described methods (230,231). Compared to cultures grown with 5 μM free Zn, cells that were grown with 25% saturated mouse calprotectin (MCP) had significantly impaired growth (**Figure 3.1**). The growth of FA19 with mCP as the sole Zn source was not statistically different from the no Zn control.

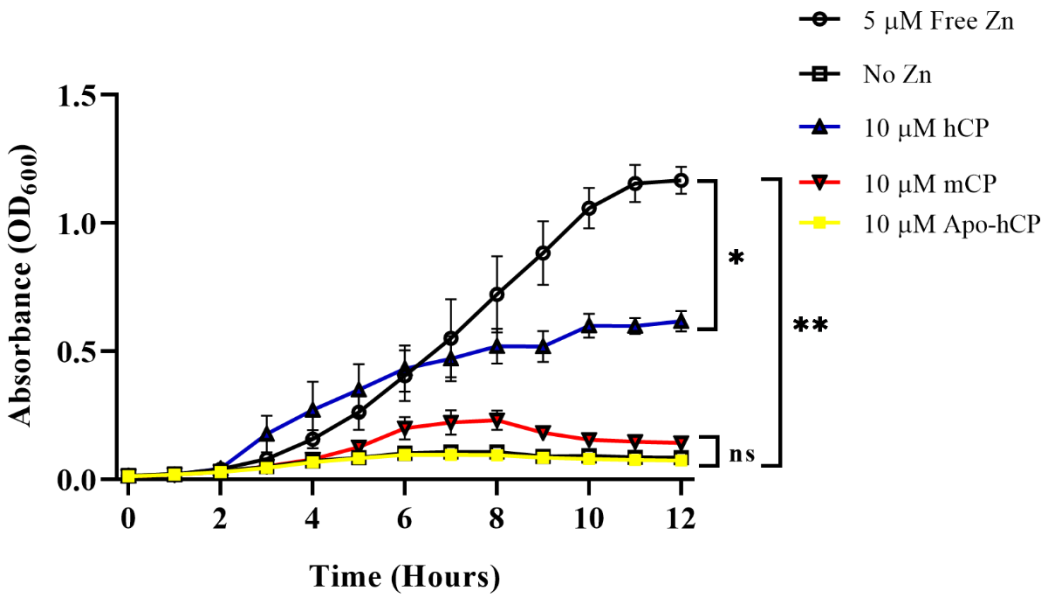

Figure 3.1 Growth of *Neisseria gonorrhoeae* when mCP is supplied as the sole Zn source.

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Gonococcal cells were allowed to double in CDM, then were diluted to an OD_{600} of ~0.02 and transferred to a 96-well microtiter plate containing concentrated growth premixes. Cells supplemented with mCP as the sole Zn source (red inverted triangles) were significantly deficient in their ability to support growth of the gonococcus beginning at 6 hours ($p<0.05$= *, $p<0.01$= **) compared to the free Zn positive control (black open circles) and hCP (blue triangles). There was no significant difference (ns) in growth between the no Zn treatment and cells supplemented with mCP as the sole Zn source. Significance was determined via a two-way ANOVA with Tukey post-test. Error bars represent standard error of the mean (SEM) of three independent experiments performed in technical triplicate.

TdfH has been implicated as being necessary for binding to hCP in whole gonococcal cells (231). In an attempt to define the specific interactions between TdfH and CP, a competition assay was developed to assess whether TdfH preferentially interacts with hCP or if mCP was also able to compete for binding to TdfH. Whole cells of the following strains were immobilized onto a nitrocellulose membrane: FA1090 (WT), MCV661 (TdfH knockout), MCV662 (TdfJ knockout), and MCV936 (TdfH/TdfJ knockout) as described in Table 1. Membrane-bound cells were blocked and probed with hCP-HRP alone, with hCP-HRP mixed plus 5- or 10-fold molar excess hCP or with hCP-HRP plus 5- or 10-fold molar excess mCP. The first row containing the WT strain FA1090 showed a decrease in the HRP signal in the presence of an unlabeled hCP competitor (**Figure 3.2A, second and third columns**). mCP competitor at either concentration did not reduce the HRP development of the blots (**Figure 3.2A, last two columns**). The second row, which contained the TdfH KO strain MCV661, showed background levels of HRP development (**Figure 3.2A**). The third row of the blot contained a TdfJ KO strain also exhibited a decrease in development when probed with hCP competitor (**Figure 3.2A, second and third columns**), but demonstrated no reduction in development when mCP was used as a competitor (**Figure 3.2A, last two columns**). The fourth and final row of the blot contained the TdfH and TdfJ double knockout (DKO) strain MCV 936, which exhibited background levels of development when probed with either hCP or mCP competitors (**Figure 3.2A**). Densitometry scans of biological triplicate competition assays were used to quantify the reduction in hCP-HRP binding to the cell surface (**Figure 3.2B**). When blots were probed with either 5- or 10-fold molar excess hCP competitor, a significant reduction in HRP signal can be observed ($p<0.05$); no significant reduction in signal was seen when mCP was added as a competitor.

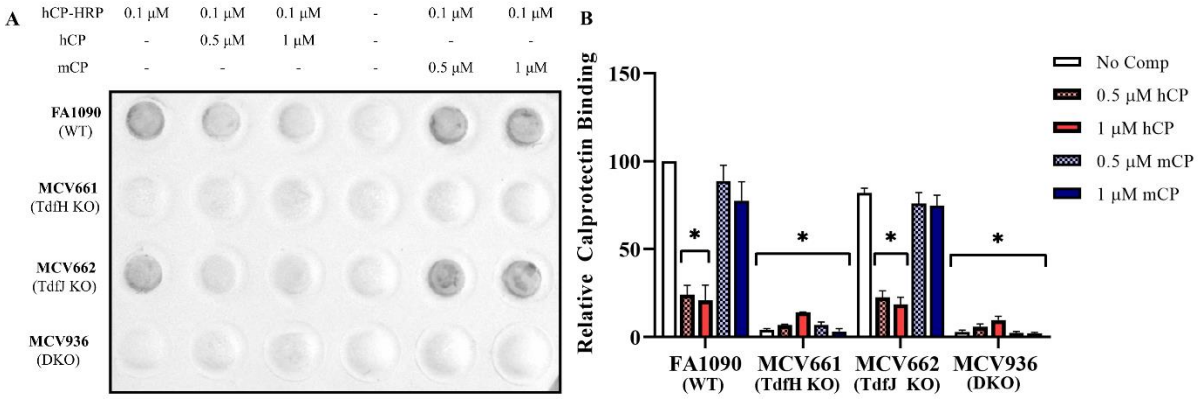

Figure 3.2 hCP and mCP competition dot blot assay.

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(**A**) Representative image of competitive CP binding assays. *N. gonorrhoeae* strain FA1090 (WT), MCV661 (TdfH KO), MCV662 (TdfJ KO) and MCV936 (DKO) were grown under Zn-restricted conditions and applied to nitrocellulose membrane at a standardized density. Membranes were probed with either 0.1 μM hCP- HRP alone or a mixture of 0.1 μM hCP-HRP combined with 0.5 μM or 10 μM hCP or mCP unlabeled competitor. (**B**) Densitometry analysis of biological triplicate sets of dot blots. Densitometry analysis was accomplished using Bio-Rad's Image Lab software. Significance was calculated via an unpaired Student's t-tests with WT-No Comp treatment used for comparison with biological triplicates done in technical triplicate (error bars calculated via the standard error of the mean (SEM), $p<0.05$ = *)

The hCP-HRP may have a higher affinity for binding to TdfH than mCP does and could, therefore, outcompete mCP for binding to TdfH. To test this hypothesis, the binding of both hCP and mCP was assayed alone independently in total CP binding assays (**Figure 3.3A**). A control western blot using the α-S100A9 monoclonal antibody was performed to ensure both hCP and mCP were detectable prior to performing the total calprotectin binding experiments. Membranes blotted with the same strains as in **Figure 3.2A** were probed with either 0.5 μM hCP or 0.5 μM mCP followed by an α-S100A9 monoclonal antibody cross-reactive for hCP and mCP. The blots were developed with an α-rabbit IgG-HRP secondary. Similar to the results from the competition assay, the WT and TdfJ KO, which both express TdfH, bound hCP but not mCP. Likewise, cells lacking TdfH (the TdfH KO and the double KO) do not bind either of the calprotectins. Quantitative measures of these blots were determined through densitometry (**Figure 3.3B**), which showed significantly reduced mCP binding (by ~60% or more) in all strains compared to hCP binding to WT. Further, all mutant strains except the TdfJ KO showed more than 60% reduction in hCP binding. The inability of mCP to interact with whole gonococci, both in a competitive format or alone coupled with Zn-saturated mCP not supporting gonococcal growth strongly suggests that mCP is not a natural ligand for TdfH. Further the lack of interaction between TdfH and mCP suggests that TdfH may have a human ligand restriction for binding and Zn-piracy.

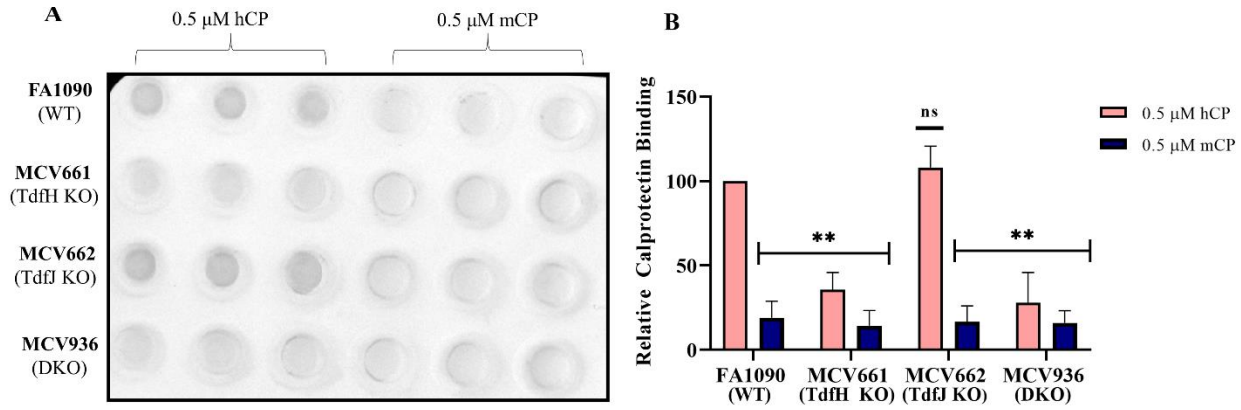

Figure 3.3 hCP and mCP binding to the gonococcal surface.

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(**A**) Representative image of direct CP binding assays. *N. gonorrhoeae* strain FA1090 (WT), MCV661 (TdfH KO), MCV662 (TdfJ KO) and MCV936 (DKO) were immobilized onto nitrocellulose were probed with 0.5 μM hCP or 0.5 μM mCP. Calprotectin bound to the surface of cells was detected with an α-S100A9 monoclonal antibody followed by detection using an α-mouse IgG conjugated to HRP. (**B**) Densitometry of three independent biological replicates performed in technical triplicate. Densitometry of scanned blots demonstrated a significant reduction in the binding of mCP to affixed gonococci compared to the binding of hCP to the WT strain. Significance was calculated by an unpaired Student's t-test with comparisons made to FA1090 probed with mCP. Error bars represent SEM with $p<0.01$ = **.

B. TdfH and hCP forms complexes detected via SEC

To determine whether we could recapitulate the TdfH interaction with hCP *in vitro*, we codon-optimized the gene sequence for TdfH and subcloned the gene into the pHIS2 and pET20b (modified with an N-terminal 10X His tag and TEV protease site) vectors for expression in *E. coli*. While the native expression (pET20b vector) was only barely observable by Western blot analysis, we were able to express TdfH into inclusion bodies (pHIS2 vector) with high yields. We refolded and purified TdfH using a Ni-NTA column and did a final purification using size-exclusion chromatography into 1x PBS with 0.05% DDM. The sample was then mixed at a ratio of 1:2 with each hCP and mCP, incubated for at least one hour, and then separated again using size-exclusion chromatography into 1x PBS with 0.05% DDM. Similarly, control samples of hCP and mCP were also analyzed for comparison. As shown in **Figure 3.4A**, we observed a clear shift in hCP such that it co-elutes with TdfH, as visualized by SDS-PAGE analysis (**Figure 3.4B**). Conversely, no observable shift was detected with mCP (**Figure 3.4C and 3.4D**), even after TCA precipitation of the samples to boost the low signal from mCP (**Figure 3.4E**). The lack of a native membrane presentation of TdfH had no effect on the interaction between TdfH and hCP. These results demonstrate that TdfH alone is responsible for the ability of *N. gonorrhoeae* to distinguish between human and mouse CP and interact with human calprotectin and.

C. hCP and TdfH interact with nanomolar affinity

One of the signature symptoms of gonococcal infection is the influx of neutrophils into the site of infection driven by localized inflammation (304-306). Human neutrophils can undergo a process of NETosis, releasing their intracellular and granular contents, including the highly abundant cytosolic protein HCP (299,300,307). hCP has been documented to reach concentrations as high as 1 mg/mL in inflamed tissues.

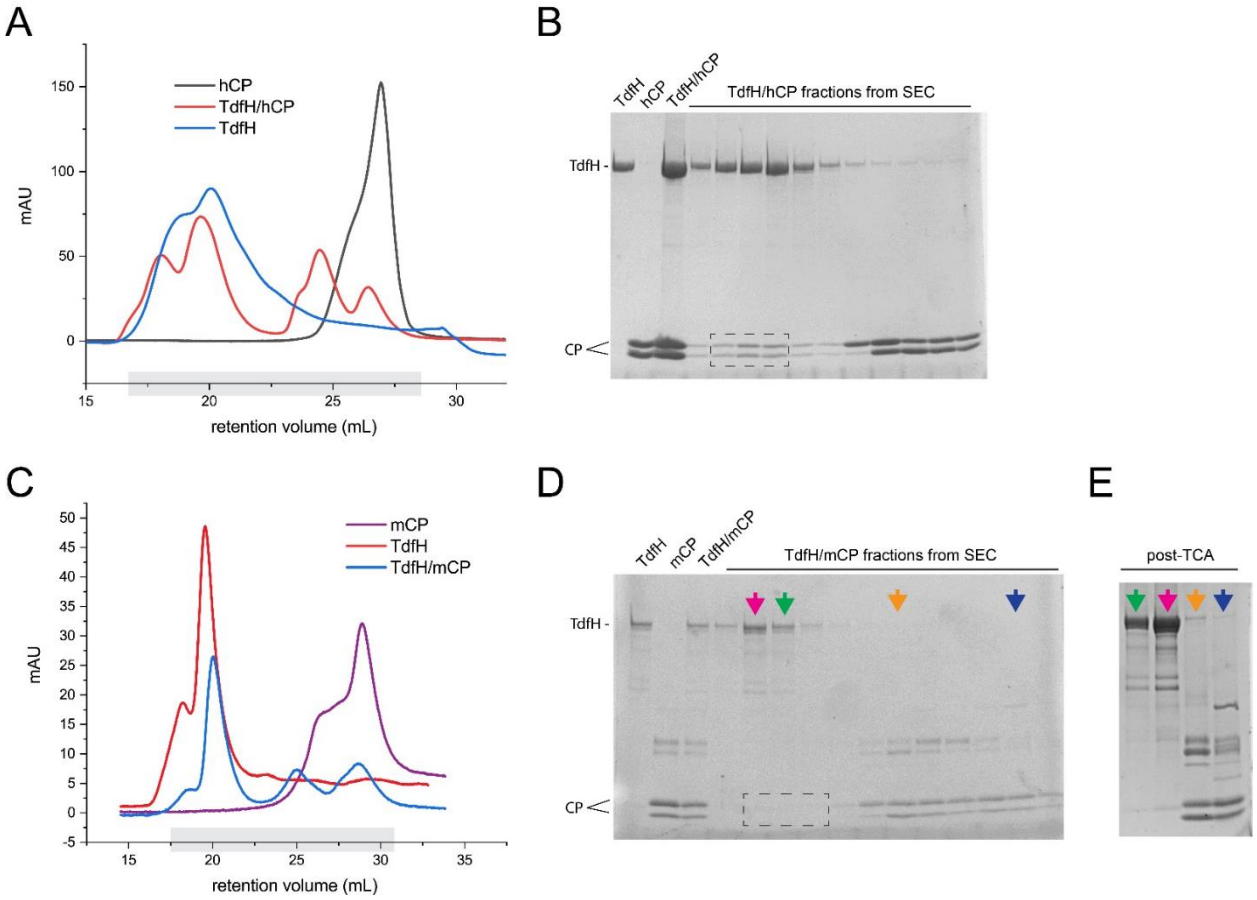

Figure 3.4 Recombinant TdfH and calprotectin complex formation.

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(**A**) Recombinant TdfH (**blue**), hCP (**black**), or TdfH incubated with hCP (**red**) were run over a Superdex 200 column. Fractions collected, which are highlighted in grey, were run on a 15% SDS-PAGE gel. (**B**) 15% SDS-PAGE of TdfH alone, hCP alone, TdfH and mCP incubated together and collected fractions. TdfH incubation with hCP resulted in co-elution in fractions collected from SEC (**dotted box**) indicating the formation of a TdfH-hCP complex. (**C**) Recombinant TdfH (**red**), mCP (**purple**), and TdfH incubated with mCP (**blue**) were run over a Superdex 200 column. Fractions collected for SDS-PAGE analysis are highlighted in grey. (**D**) 15% SDS-PAGE gel of TdfH, mCP and collected fractions of TdfH incubated with mCP. TdfH and mCP that had been incubated together independently eluted during SEC (**dotted box**). (**E**) Two fractions that eluted only TdfH (**purple and green arrow**) and only mCP (**orange and blue arrow**) were chosen for TCA precipitation to see if any amount of TdfH or mCP eluted together. TCA precipitated contained only TdfH (**purple and green**) or mCP (**orange and blue**) indicating the inability for TdfH-mCP complexes to form.

We therefore investigated whether this high abundance neutrophil protein, found on inflamed mucosal membranes, interacted with TdfH with high affinity. Isothermal titration calorimetry (ITC) was used to determine the binding affinity of the interaction between TdfH and hCP and duplicate experiments were performed to ensure that the data was reproducible. Prior to injections of CP into TdfH, hCP and mCP were titrated into a sample cell containing just buffer. These injections determined that hCP and mCP had a minimal heat release when injected into buffer alone, and thus our titrations were optimized to reach a phase of static heat release upon ligand saturation rather than no heat release which is typical of ITC isotherms. Here, 300 μL of 20 μM of TdfH was loaded into the sample well of a Nano ITC microcalorimeter, and 2.5 μL of 200 μM of hCP was incrementally titrated into the TdfH over 20 injections. Using the NanoAnalyze software package, the isotherm of the TdfH and hCP ITC experiment (**Figure 3.5A**) was found to best fit a two-state model, allowing us to determine the binding parameters for the interaction. Two distinct binding profiles were observed indicating multiple modes of interaction in a two-state model. The first was a high-affinity interaction with the affinity calculated to be 4.0 nM (**Table 2**). The second binding profile was a low-affinity interaction with an affinity 35 μM. The lack of growth support, competition, and complex formation with TdfH by mCP led us to question if mCP had any detectable interaction with TdfH. Similar to the ITC of hCP, a titration of mCP was performed and analyzed using a two-state model (**Figure 3.5B**). Similar to the addition of hCP, the titration of mCP into the TdfH containing cell yielded two affinities, with the highest affinity determined to be 0.72 μM and the lowest as 51 μM (**Table 2**). However, unlike the hCP interaction there was less overall heat release (kcal/mol) when mCP was added to TdfH indicating that there was minimal interaction between mCP and TdfH. The ITC experiments determined that TdfH binds to hCP with high affinity, while the interaction of

TdfH and mCP is much lower. The ITC experiments also found that there was a second unique binding interaction between TdfH and CP. The two-state interactions suggest that TdfH may bind to CP at two unique sites where one of those sites is the preferred location for Zn metal piracy, or TdfH could bind to CP in two stages.

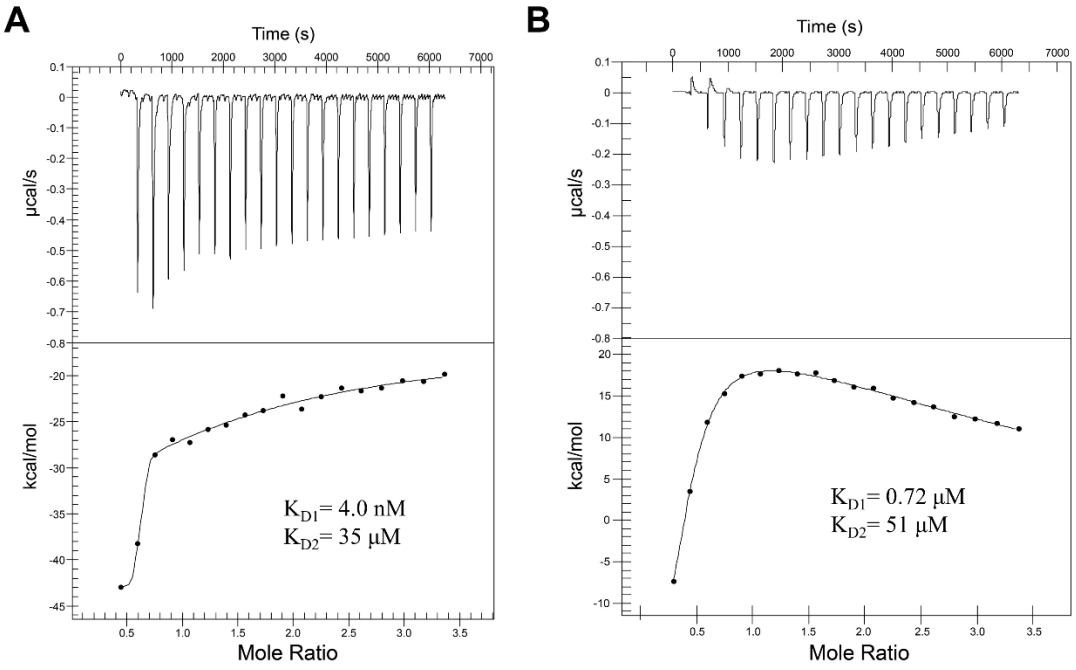

Figure 3.5 Isothermal titration calorimetry of hCP and mCP with TdfH

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Calprotectin was titrated into TdfH over 20 injections. ITC experiments were done in duplicate to confirm reproducibility of the isotherm fits. (**A**) Using NanoAnalyze, the isotherm of the hCP injections best fit a multi-site model and resulted in high and low affinities of 4.0 nM and 35 μM (bottom). When hCP was incrementally titrated into the TdfH containing sample well, a robust heat release was detected (top). (**B**) The mCP titration isotherm had dramatically reduced kcal/mol heat release compared to hCP (top). The mCP isotherm best fits a two-state model with calculated high and low affinities of 0.72 μM and 51 μM (bottom).

Best-fit	Multi-site	Multi-site	Multi-site	Multi-site	Independent
Model Ka_1 (M^{-1})	$2.5 \times 10^8 \pm 4.7 \times 10^7$	$1.4 \times 10^6 \pm 3.4 \times 10^6$	$8.6 \times 10^5 \pm 3.8 \times 10^6$	$1.5 \times 10^7 \pm 1.5 \times 10^7$	-
Ka_2 (M^{-1})	$2.8 \times 10^4 \pm 5.9 \times 10^3$	$2.0 \times 10^4 \pm 5.6 \times 10^4$	$1.5 \times 10^4 \pm 9.2 \times 10^4$	$1.7x\ 10^4 \pm 1.8 \times 10^4$	-
n_1	0.55 ± 0.017	0.28 ± 0.063	1.3 ± 0.208	0.40 ± 0.012	0.67 ± 0.12
n_2	1.2 ± 0.5	7.8 ± 2.2	2.1 ± 6.4	8.8 ± 1.0	-
ΔH_1 (kcal/mol)	-26 ± 1.7	-50 ± 19	3.2 ± 17	-4.6 ± 5.9	-25 ± 5.04
ΔH_2 (kcal/mol)	-32 ± 4.7	50 ± 7.4	50 ± 20	25 ± 13	-
K_{D1} (M)	4.0×10^{-9}	7.3×10^{-7}	1.2×10^{-6}	6.6×10^{-8}	$1.3 \times 10^{-5} \pm 3.8 \times 10^{-6}$
ΔS_1 (cal/mol·K)	-50	-140	38	17	-62
K_{D2} (M)	3.5×10^{-5}	5.1×10^{-5}	6.8×10^{-5}	6.0×10^{-5}	-
ΔS_2 (cal/mol·K)	-81	190	190	101	-

Table 2 Summary of isothermal titration calorimetry parameters

D. hCP and mCP share limited sequence identity

The ability for TdfH to differentiate between the human and mouse forms of CP led us to investigate the similarity between the amino acid sequences of the proteins. CP is an obligate heterodimer made up of S100A8 and S100A9, and contains two metal-binding sites of interest which have been previously identified as site 1 and site 2 (**Figure 3.6**) (308). The sequences for human S100A8 (AAH05928.1) and S100A9 (AAH47681.1) were aligned to mouse S100A8 (NP_038678.1) and S100A9 (NP_001268781.1) using Geneious software and then visualized using ESPript 3 (Figure 6B) (194,309). Human and mouse S100A8 shared 58% sequence identity and 83% similarity, while the S100A9 proteins shared 58% sequence identity and 74% similarity. Mapping the divergent residues to the surface of the hCP structure (**PDB ID 4GGF**) reveals that most of the diversity is found at the ends of the CP structure in proximity to the site 1 and site 2 metal-binding sites (**Figure 3.6C**).

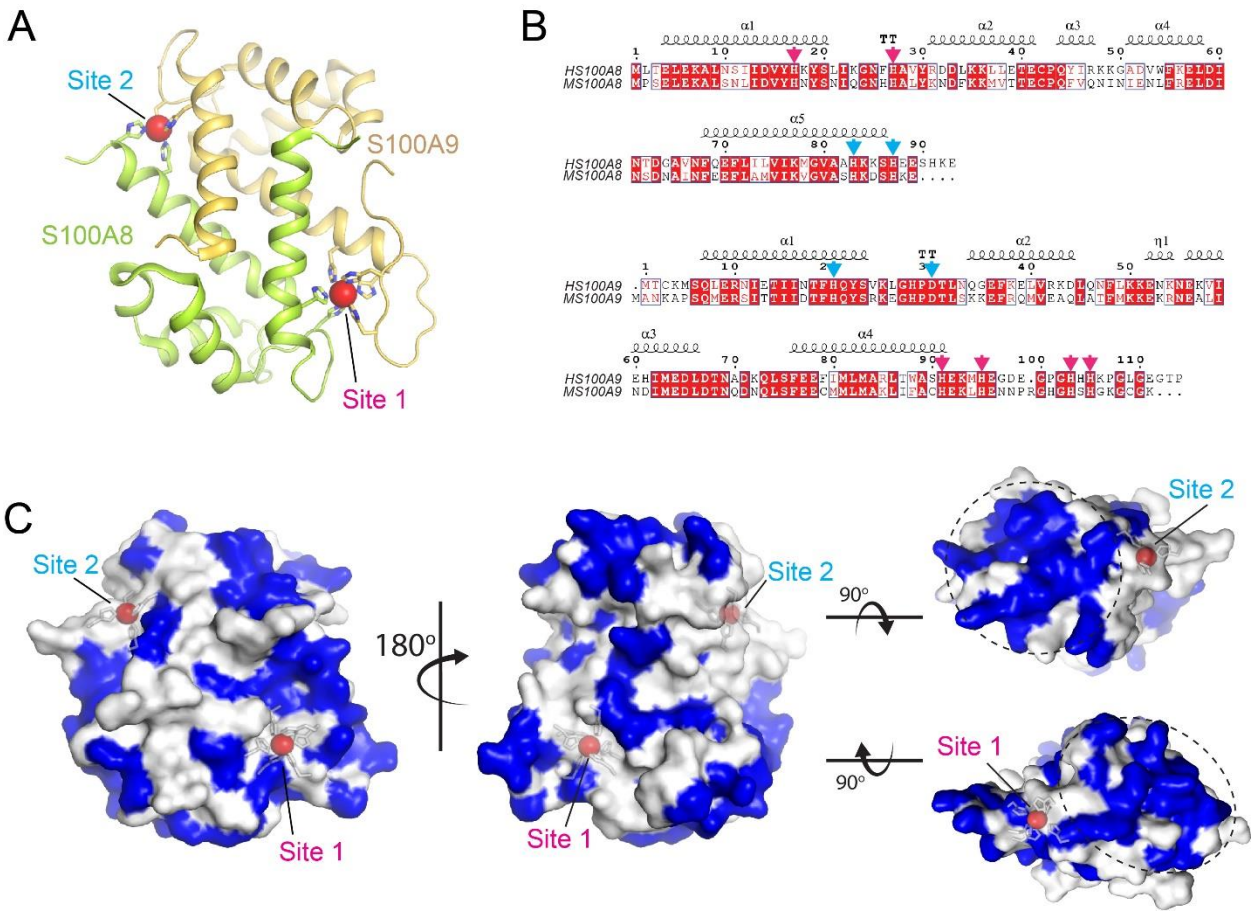

Figure 3.6 Pairwise alignment of human and mouse S100A8 and S100A9 proteins

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(**A**) The structure of human calprotectin (**PDB ID 4GGF**) in complex with manganese (**red spheres**). The locations of metal-binding sites 1 and 2 are indicated in stick. (**B)** A sequence alignment of human and mouse calprotectin subunits, with mapped secondary structural elements. Sequences for human (**AAH05928.1**) and mouse (**NP_038678.1**) S100A8 proteins were aligned through Geneious with a BLOSUM 65 matrix. The S100A8 sequences share 58% identity and 83% sequence similarity. Mouse S100A8 is 89 amino acids whereas human S100A8 is 93 amino acids in length. Sequences for human (**AAH47681.1**) and mouse (**NP_001268781.1**) S100A9 proteins were aligned sharing 58% identity and 74% sequence similarity. Mouse S100A8 is 113 amino acids whereas human S100A8 is 114 amino acids in length. Residues making up the site 1 metal-binding site are indicated by the magenta arrows while those making up the site 2 metal-binding site are indicated by cyan arrows. (**C)** Based on the sequence alignment in panel B, residues that are different between human and mouse calprotectin were mapped to the surface of the human calprotectin structure (highlighted in blue; gray indicates identical residues). The most divergent regions between the two are found along the ends of the calprotectin structures close to the site 1 and site 2 metal-binding sites (**indicated by the dashed circles).**

E. The S1KO hCP is unable to support the growth of *N. gonorrhoeae*

The antimicrobial properties of HCP have been found to negatively affect a variety of microorganisms, including *Staphylococcus aureus* and *Candida albicans* (202,203,277). This antimicrobial effect has been attributed to the metal sequestration properties of the protein. hCP has two sites available for metal sequestration, with site 1 capable of coordinating a Zn or Mn ion, and site 2 coordinating a Zn ion only (204). To determine whether TdfH was capable of Zn acquisition from both metal-coordinating sites of hCP, FA19 was grown with the following as the sole Zn source: WT hCP, site 1 knockout CP (S1KO), site 2 knockout CP (S2KO), and a total knockout CP which binds no metals (TKO), which were all purified and supplied by Dr. Walter Chazin from Vanderbilt University (**Figure 3.7**). The S1KO has Asn substitutions at the four His residues responsible for transition metal binding. The S2KO has Asn substitutions at the three His residues and a Ser substitution at the Asp residue responsible for transition metal binding. The TKO has both of the above mutations. Calprotectin added to the concentrated growth premixes was previously loaded with $ZnSO_4$ to achieve 25% saturation (230,231). Cells that were grown in the presence of the S1KO as the sole Zn source demonstrated limited growth, similar to that of the no Zn negative control. Similarly, gonococci grown in the presence of the TKO grew in a manner that was not significantly different from the no Zn control. Gonococci grown with S2KO as the sole Zn source, by contrast, demonstrated growth patterns that were not significantly different from cells grown using the WT hCP. Free Zn, WT hCP, and the S2KO CP all supported growth that was significantly higher when compared to the no Zn, S1KO and TKO HCP controls. No significant differences were noted in the growth patterns when gonococci were provided free Zn, WT hCP, or S2KO as sole Zn sources. The lack of growth support detected

with the S1KO provides strong evidence that the interaction between TdfH and hCP is located predominantly around site 1 and is potentially the primary location of Zn piracy from hCP by the gonococcus.

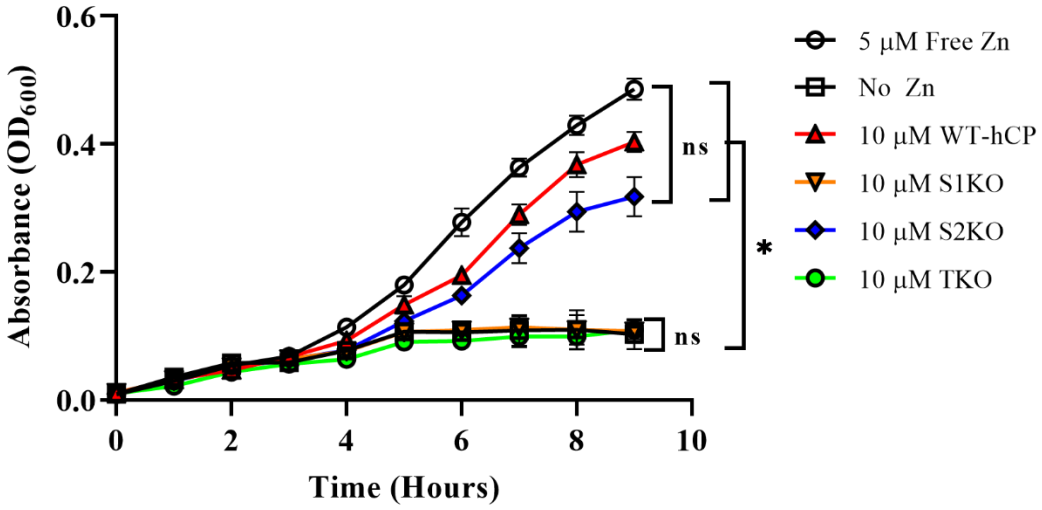

Figure 3.7 Growth of *N. gonorrhoeae* when hCP Zn-site knockouts are used as the sole Zn source.

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Gonococcal strain FA19 was grown with concentrated premixes containing hCP and hCP site knockouts as the sole Zn source. hCP was saturated to 25% with $ZnSO_4$ and dialyzed overnight against their native buffer containing chelex-100 resin to remove any unbound residual Zn. FA19 that had been grown with 5 μM free Zn (black open circles), 10 μM WT-hCP (red triangles) and 10 μM S2KO (blue diamonds) had significantly increased growth compared to the No Zn treatment (back open boxes) and had no significant difference in growth when compared to each other. The free Zn, WT, and S2KO also demonstrated a significant growth increase compared to the S1KO (orange inverted triangles) and TKO (green circles). The S1KO and TKO growth were not significantly different from the growth of our no Zn negative control which displayed minimal growth over the 9-hour incubation. Statistics of biological triplicate performed in technical triplicate were done via a two-way ANOVA with a Tukey post-test. $p<0.05$= *. Error bars represent standard error of the mean (SEM).

F. TdfH interaction with hCP depends on the sequence at each Zn binding-site

ITC was employed in order to examine the role that each separate metal-binding site of hCP plays in the interaction with TdfH. TdfH was mixed with either S1KO or S2KO over 20 injections as previously described in this study. Isotherms for the S1KO and S2KO were analyzed with NanoAnalyze and the best fit was to a two-state model. Similar to the wild-type protein, both the S1KO and the S2KO mutated proteins demonstrated two distinct binding affinities (S1KO: 1.2 μM and 68 μM [**Figure 3.8A**]; and S2KO: 66 nM and 68 μM [**Figure 3.8B**]). Taken together, these results suggest that knocking out site 1 has a more negative effect on HCP binding than does knocking out site 2. Further, we also titrated in hCP-TKO with TdfH (**Figure 3.8C**). Interestingly, analysis of the TKO data showed a shift from a multi-site model to an independent model with a single, low-affinity binding of 13 μM. These data align well with the cell-based studies presented in the previous section where we demonstrated that site 1 appears to be more important for supporting growth of the gonococcus (**Figure 3.7**).

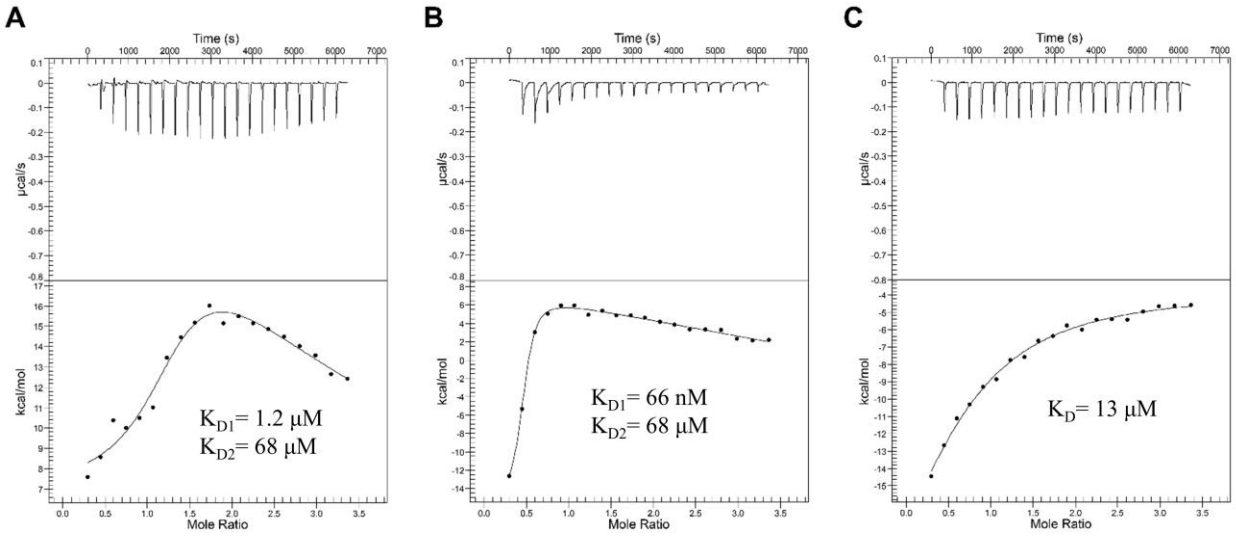

Figure 3.8 Isothermal titration calorimetry of S1KO, S2KO, and TKO hCP with TdfH

Figure 3.8 Isothermal titration calorimetry of S1KO, S2KO, and TKO hCP with TdfH

The S1KO HCP (**A**) and S2KO HCP (**B**) were each titrated into TdfH over 20 injections. Isotherms for both the S1KO and S2KO titrations best fit with a multi-site model using NanoAnalyze. **A**. Injection of S1KO into TdfH was calculated to have high and low affinities of 1.2 μM and 68 μM. **B**. Injection of S2KO into TdfH was calculated to have high and low affinities of 66 nM and 68 μM. **C**. The isotherm for the TKO titrations best fit an individual site model. Injection of TKO into TdfH was calculated to have an affinity of 13 μM. The S1KO, S2KO, and TKO isotherms all had dramatically reduced kcal/mol heat release compared to hCP.

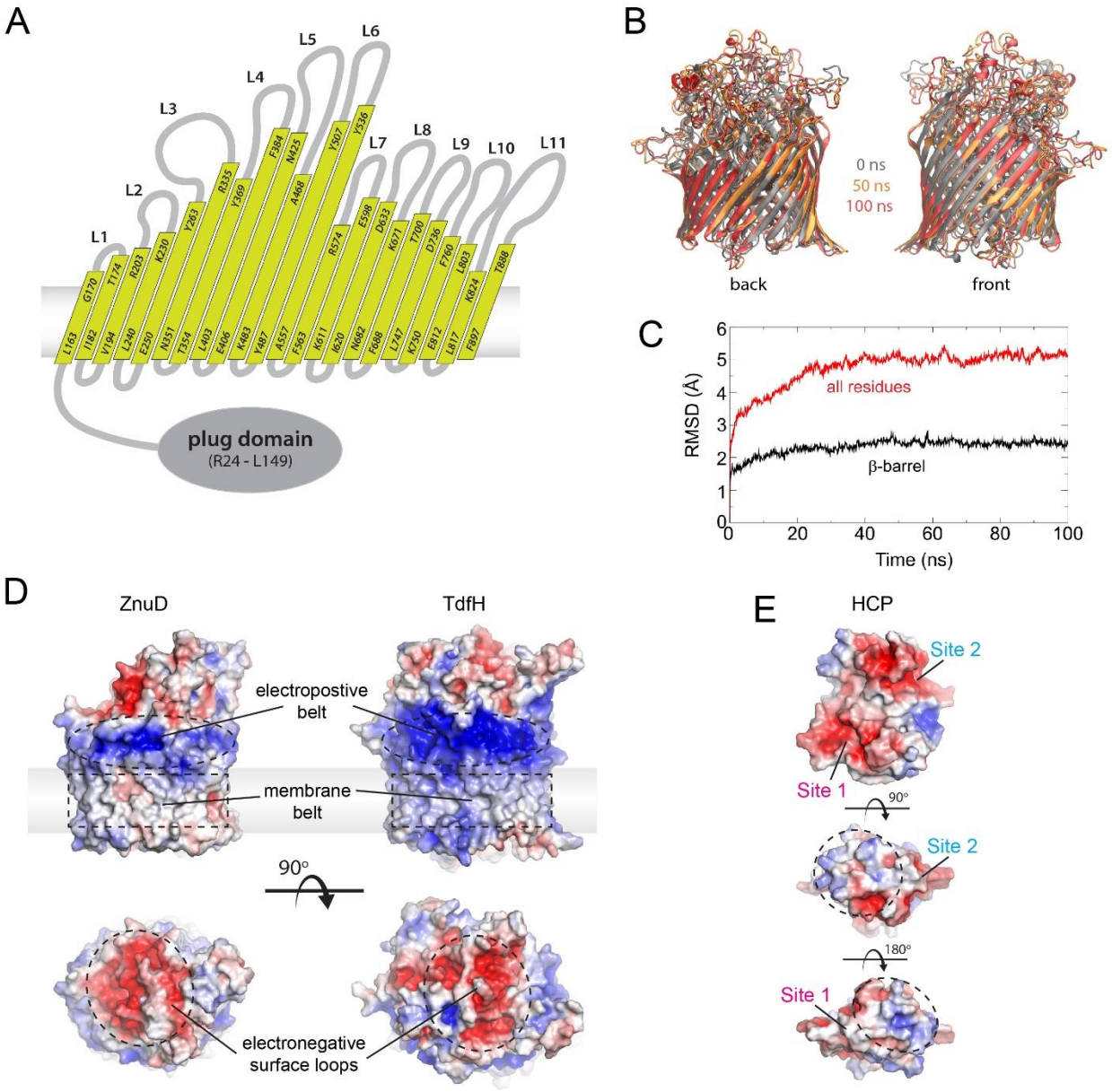

Figure 3.9 Homology model of TdfH and insight into the interaction with human calprotectin

Figure 3.9 Homology model of TdfH and insight into the interaction with human calprotectin.

(A) The membrane topological map of TdfH based on homology modeling using sequence and structural alignments of the closest homologs. (**B)** A superposition of the TdfH models from the molecular dynamics (MD) simulations at 0, 50, and 100 nanoseconds (gray, gold, and red, respectively), showing a stable membrane barrel domain throughout the simulation with little variability. (**C**) A plot from the MD simulations of the average RMSD of TdfH residues within the barrel only versus all residues. The barrel domain (as well as the plug domain) is significantly more stable than the rest of the protein which consists mostly of elongated extracellular loops. (**D**) Electrostatic surface potential comparison of the ZnuD structure (**PDB ID 4RDR**) with the TdfH homology model. Similar characteristics were observed, including the electropositive belt (**dashed ovals**), the membrane belt (**dashed rectangles**), and the electronegative surface loops (**bottom, dashed circles**). (**E**) Electrostatic surface properties of human calprotectin (**PDB ID 4GGF**). Both site 1 and site 2 regions were observed to be strongly electronegative, while the divergent regions noted in Figure 6 in proximity to site 1 and site 2 were significantly less charged by comparison. A nearly identical observation was seen with a model of mouse calprotectin (data not shown), suggesting that electrostatics alone is not responsible for binding to TdfH.

Discussion

Neisseria gonorrhoeae is a pathogen that is uniquely adapted for survival inside the human body. An evolutionary study examining primate orthologs of transferrin found that transferrin, specifically the C lobe that interacts with neisserial TbpA, has undergone a rapid evolution, driven by positive selection by bacterial TbpA proteins (310). This TbpA-driven transferrin evolution emphasizes the impact that gonococcal iron piracy proteins have on their host (311). While much of nutritional immunity was previously focused on the sequestration of iron, the importance of controlling the concentrations of other free transition metals, such as Zn, Mn, and Cu, has recently been recognized (203,291,308,312-314). The S100 family of proteins, all of which are EF-hand calcium-binding proteins, has been implicated as one of the major transition metal chelators in inflamed tissues (193,194). Two TdTs produced broadly by the pathogenic *Neisseria* have been found to utilize select S100 proteins as sole metal sources in order to overcome host nutritional immunity. The TdfH homolog, renamed CbpA, was demonstrated to enable interaction between *Neisseria meningitidis* and CP (302). Jean et al. subsequently demonstrated that *N. gonorrhoeae* could grow on CP and internalize Zn from CP in a TdfH-dependent manner and that production of TdfH enhanced gonococcal survival in neutrophil NETs (231). The TdfJ homolog, renamed ZnuD, was purified from *N. meningitidis* and crystalized. The structure of this TdfJ homolog bound directly to Zn and thus was described as a Zn transporter (229,315). Subsequently, Maurakis et al. determined that TdfJ enabled growth and Zn uptake from an S100 protein, S100A7. This study further demonstrated that TdfJ specifically bound to the human form of S100A7 and not the mouse protein (230).

Calprotectin is an obligate heterodimer of two S100 proteins, S100A8 and S100A9, and is found at high concentrations within the cytoplasm and the granular contents of neutrophils and

monocytes (316,317). Calprotectin has been documented to bind to Zn, Mn, Cu, and Fe, which contributes to its antimicrobial properties against a variety of pathogens (202-204,277,318). Calprotectin is also abundant within NETs (299). Interestingly, *N. gonorrhoeae* modulates the immune environment to upregulate a Th-17 and T-regulatory immune response and downregulate a Th-1/Th-2 immune response. This Th-17 response drives a large influx of neutrophils to the site of infection resulting in the classical purulent discharge associated with gonococcal infection (57,319). *N. gonorrhoeae* is adept at surviving this influx of neutrophils and their toxic effects. *N. gonorrhoeae* can inhibit the processes of phagocytosis, phagosome maturation, and reduce the production of reactive oxygen species through the expression of surface proteins (304). The phase variable nature of gonococcal LOS also allows for increased survival in the presence of cationic peptides, which are also abundant within NETs (58). NET production involves an irreversible expulsion of intracellular contents, which trap invading pathogens in a hostile and toxic environment. However, *N. gonorrhoeae* is capable of surviving NETs in part due to the production of a thermonuclease and the TdT, TdfH (231,298,307,320).

The most well-characterized, gonococcal metal transport system is comprised of TbpA and TbpB, which are responsible for high-affinity transferrin binding and iron piracy. TbpA, like other TdTs, has an N-terminal plug domain that contains a TonB box, and C-terminal β-barrel (210). TbpA also demonstrates a host restriction for human transferrin (hTf) and is incapable of binding to a variety of animal transferrins (214,321). The co-crystal structure of *N. meningitidis* TbpA and hTf showed that a large surface area of TbpA (~2500 $Å^2$) interacts with hTf. Further, the co-crystal structure revealed an α-helix within one of the extracellular loops of TbpA that was in close association with the iron-binding cleft of the C-lobe of hTf (210). Investigations into

the function of the loop 3 helix found that a complete deletion of the structure abrogated hTf binding and subsequent iron internalization (281).

Conservation of the gonococcal TdT systems has led to our hypothesis that other TdTs share similar structural and functional relationships with their ligands as TbpA and TbpB have for hTf. LbpA, which allows for iron acquisition, from human lactoferrin (hLf), resembles TbpA structurally as it also contains an N-terminal plug domain with a TonB-box and a 22 stranded C-terminal β-barrel (217,322). Further, only expression of LbpA is required for iron acquisition from hLf, and the expression the cognate lipoprotein LbpB, like the hTf system, makes iron acquisition more efficient by distinguishing between apo-and holo- lactoferrin (216,217). Interestingly, neither the TdfH nor TdfJ systems have a companion lipoprotein associated with them (295). The S100 protein ligand for TdfH is able to bind both Zn and manganese (Mn^{2+}) and both hCP and S100A7 are present in high abundance in inflamed tissue (194,204,323) . Therefore, a cognate lipoprotein that distinguishes between apo- or metal-loaded ligand may not provide a benefit in either of these Zn systems. Both the TbpAB and LbpAB systems have been shown to be human-restricted for their ligands and consequent iron internalization (252,324). This commonality between TdTs is not seen only with the iron transporters but also with the Zn transporters. The crystal structure of ZnuD, the meningococcal homolog of TdfJ, also shows structural similarity, including a loop 3 helix similar to that of TbpA (315). The study into the interaction between TdfJ and S100A7 of *N. gonorrhoeae* found the interaction to be human specific (230).

The binding between TdfH and CP has previously been demonstrated only in the context of whole cells and thus, it was formerly possible that other membrane factors could influence or be responsible for the interaction (231). ITC experiments in the current study (**Figure 3.5, Table**

2) were performed and allowed us to identify a unique pattern of interaction between TdfH and CP with two binding affinities. The first interaction between WT-hCP and TdfH was determined to be a high-affinity interaction (4.3 nM) and the second was lower affinity at 38.3 μM. In contrast, the TdfH-mCP interactions were measured as 0.4 μM and 25.6 μM, both lower than the high-affinity interaction seen with TdfH-hCP. The dimer of CP is capable of coordinating a Zn atom at either site 1 or site 2 (133,136,152). Since hCP is a heterodimer, the two-state interaction and particularly the high affinity interaction between hCP-TdfH may be due to a preference of TdfH for either site 1 or site 2, which are present on opposite sides of the dimer interface.

N. gonorrhoeae is an obligate human pathogen with a demonstrated species restriction for interaction between host proteins and gonococcal surface proteins including the TdTs (230,252,325). The competition assay between hCP and mCP binding (**Figure 3.2**) demonstrated no competition by mCP; however, this could have been due to the difference in the affinity of the hCP-TdfH and mCP-TdfH interactions (**Figure 3.55, Table 2**). When mCP and hCP were used independently to directly probe the surface of *N. gonorrhoeae* (**Figure 3.3**), hCP bound to strains possessing TdfH (FA1090 and MCV662) as has been described in both *N. meningitidis* and *N. gonorrhoeae* (231,302), while mCP binding was undetectable. Analysis of the amino acid sequence diversity between human and mouse S100A8 and S100A9 found that both shared 58% sequence identity and they shared 83% and 74% amino acid sequence similarity, respectively. The observation that evolution of hTf has been driven in part by TbpA positive selection may explain the sequence diversity found between human and mouse S100A8 and S100A9 (310). Evolution hCP may have occurred through a similar positive selection, which has been driven by TdfH binding to host ligand.

The Zn-binding sites of CP are formed at the dimer interface; however, S100A8 contributes 4 of the 6 residues to the non-canonical binding site (site 1, His6), while S100A8 and S100A9 contribute an equal number of residues to the canonical binding site (site 2, His3, Asp) (**Figure 3.6**) (194,204,206,301). hCP with mutations in either metal-binding site 1 (S1KO) or site 2 (S2KO) were used to test whether these residues contributed to the ability of TdfH to interact with CP and whether these sites were utilized equally for Zn piracy (**Figure 3.7**). Growth of *N. gonorrhoeae* was significantly hindered when Zn-binding was abrogated in site 1. When the S1KO was titrated into purified TdfH, an isotherm with minimal heat release was seen, along with two micromolar interactions. The S2KO, by contrast, resembled the WT-HCP isotherm with one nanomolar interaction and one micromolar interaction; however, mutation of site 2 did reduce the affinity ~15 fold. S2KO CP was capable of supporting the growth of the gonococcus when provided as the sole Zn source. The difference in affinity and the ability to support the growth of the gonococcus suggests that the absence of the non-canonical site (S1) of hCP significantly hinders TdfH binding and use as a Zn source. Mutation of the canonical site (in the S2KO) had a minimal impact on the overall affinity compared to WT-CP (**Figure 3.8**). Taken together, these data suggest that the region around site 1 may be the primary interaction site between TdfH and hCP.

In lieu of a TdfH crystal structure, we used extensive sequence and structural modeling to form a homology model of TdfH, which offers clues for how it may interact with hCP (**Figure 3.9**). To validate the model itself, we did molecular dynamics simulations, which show a stable model over the course of 100 nanoseconds within a membrane bilayer. Further, electrostatic surface maps of the TdfH model reveal clear domain separation of the membrane belt and also displays properties that align well with previously-crystalized meningococcal ZnuD, including

an electropositive belt along the surface, in proximity of the membrane domain, and strongly electronegative surface loops (**Figure 3.9B**). We also analyzed the electrostatics of hCP, finding that the site 1 and site 2 regions were predominantly electronegative, making it unlikely that those directly participate in binding at the surface loops. Rather, regions encompassing sites 1 and 2 may interact along the electrostatic belt. Interestingly, the divergent ends of hCP, which we hypothesize may contribute most to the interaction between hCP and TdfH, were not strongly charged (**Figure 3.9C**). Our homology model will allow for future studies that interrogate the exact contributions that loops of TdfH have on the interaction with hCP. Understanding how the TdfH loops contribute to hCP binding will also aid in directed-mutagenesis studies to abrogate ligand binding to produce a better antigen for eventual inclusion into a vaccine for the prevention of *N. gonorrhoeae* infection, similar as was seen with mutants of TbpB in *H. parasuis* therapies (326). In that study, point mutations were generated that abrogated TbpBs ability to bind to porcine Tf. The abrogation of ligand binding enables the immune system to have larger area for antigen recognition. Furthermore, the native proteins are still capable of binding to its host ligand and can effectively mask itself from the immune system by looking like the host. When these mutant TbpBs were used to immunize piglets against *H. parasuis*, they produced a more robust immune response compared to native TbpB, and protected piglets from a lethal dose infection. Frandoloso *et al.* provided strong evidence to confirm the hypothesis that abrogating ligand binding does produce more potent immunogens (326). Applying these directed mutagenesis studies to gonococcal TdTs could increase their potency as a vaccine antigen.

Our study has provided a detailed view of the unique interaction between bacterial and host proteins. Identification of a species preference for CP informs the need to generate an hCP transgenic mouse model for further *in vivo* function and immunogenicity testing. Moreover, the

finding that site 1 of hCP may be the preferential site of Zn piracy will further guide mutagenic experiments aimed at deciphering the molecular mechanism used by TdfH to mediate gonococcal virulence. Performing similar methods on TdfH to what has been done to mutate TbpA (281), could define the exact residues that contribute to binding and Zn-piracy of hCP.

Chapter 4: The Effect of Iron Stress on Antimicrobial Sensitivity and Abrogation of Transferrin Binding Through TbpA Mutational Analysis and Small Molecule Inhibitors

I. Introduction

Despite iron being one of the most abundant elements on earth, and an essential component for the oxidative respiration of living organisms, the human body is almost devoid of bioavailable iron. Host derived metalloproteins or hemoproteins, including transferrin, lactoferrin, and hemoglobin, bind the majority of free iron in the body (293,327). Sequestration of iron increases in an inflammatory state due to the body's hypoferremic response that induces the production of more lactoferrin at mucosal surfaces and reduces transferrin saturation from 30% to as low as 5% (293,328). The host's restriction of iron has been classically described as host "nutritional immunity" and has now been extended to include zinc and manganese restriction (129,291). Microbes can overcome this extreme metal deprivation during host colonization by several strategies including the production and secretion of low molecular mass iron-binding siderophores or direct removal of metals from host metalloproteins known as metal piracy (224,329-331).

Members of the Neisseriaceae family utilize a unique strategy to overcome host nutritional immunity involving the direct binding of host proteins and the removal of their iron cargo (294). For example, in *N. gonorrhoeae,* the transferrin iron uptake system binds the host glycoprotein transferrin through a bipartite receptor, which facilitates the removal and internalization of iron (209). This transferrin binding and iron uptake system consists of two proteins, TbpA and TbpB (215,321). TbpA is a TonB-dependent outer-membrane transporter that utilizes the proton motive force and the TonB, ExbBD complex for iron import into the periplasm (209,232,332). TbpB is a bilobed lipoprotein that selectively binds holo-human

transferrin (hTf) and increases the efficiency of iron uptake from transferrin. In the gonococcal genome *tbpB* is encoded upstream of *tbpA* in a bicistronic operon (332-334). Additional iron uptake systems in the gonococcus are responsible for binding to other host-derived iron-binding proteins including lactoferrin and hemoglobin, via LbpAB and HpuAB, respectively (295,303,335). The lactoferrin system is only encoded by approximately 50-70% of strains due to a deletion in *lbpB*, and the hemoglobin system is subject to subject to slipped-strand mispairing and is phase off in 70% of isolates (218,336). The transferrin system of *N. gonorrhoeae* has been found in 100% of isolates recovered, demonstrates limited antigenic variation and is not subject to phase variation, which makes it a promising candidate for the generation of a cross protective vaccine or target for disease treatment (296). An isogenic deletion mutant of *tbpA* was incapable of establishing an infection in a human male volunteer infection study, suggesting the transferrin binding system is essential for host colonization/ persistence (257). Given the phase variable nature or complete absence of other iron transport systems in the gonococcus, the transferrin system, specifically TbpA, provides a possible target for future vaccine development and potential treatments of gonococcal infection. The dramatic rise of antibiotic resistance found among gonococcal isolates has pushed the classification of *N. gonorrhoeae* to an urgent threat by the CDC and WHO (60). The current CDC recommended treatment is dual therapy of 1 g oral azithromycin and intramuscular ceftriaxone (62). However, the emergence of high levels of resistance to both azithromycin and ceftriaxone have resulted in treatment failures, highlighting our need for developing new treatments/ preventative measures for gonococcal disease (61,286).

Mutational studies on the homologous transferrin system of *Haemophilus parasuis* show that the abrogation of TbpB's ability to bind transferrin resulted in increased immunogenicity

and protection from lethal dose infection in a piglet model. Individual residues of *H. parasuis* TbpB were mutated, resulting in non-binding mutants used to immunize piglets before a lethal dose infection. Surprisingly, piglets immunized with the non-binding mutant forms of TbpB demonstrated a 100% survival rate compared to the 25% survival rate seen when a commercially available vaccine for *H. parasuis* was used (326). This study illustrated the efficacy of using mutagenized proteins abrogated in their binding capabilities to elicit a robust and functional immune response. Since the Frandoloso study was based on the analogous transferrin uptake system, it was of interest to us as it may allow for the usage of the gonococcal transferrin system as a vaccine target.

Gonococcal TbpB is highly variable and is approximately 65% similar across strains. Meningococcal TbpB has been characterized as two distinct subtypes, which can vary in size from 65 kDa – 85 kDa (337). Due to variability in size and sequence of gonococcal TbpB proteins, the less variable and more conserved TbpA was selected for mutational analysis. An alpha-helix secondary structure within loop 3 known as the loop 3 helix (L3H) illustrated the importance of this loop structure for the binding and iron internalization of TbpA. This study highlights the role that secondary structures of TdTs, and the amino acids within, have for both ligand binding and metal piracy (281).

The co-crystal structure of *N. meningitidis* TbpA bound to hTf has allowed for the design of new methods for treating gonococcal infection via mapping the intimate interactions that enable TbpA binding (210). One such method is the use of small molecules that interfere with protein-protein interactions at the interface between hTf and TbpA. A strain unable to utilize lactoferrin as an iron source and not possessing a functional hTf-system was incapable of colonizing and establishing an infection during a human male infection study (212). Thus,

screening databases for small molecules that inhibit the protein interactions between TdTs and their ligands can prove to be a powerful potential treatment for gonococcal disease. This study investigates additional mutagenesis of the TbpA loop 3-helix and several other TbpA residues hypothesized to contribute to the TbpA and hTf interaction Additionally this study investigates the efficacy of small molecule inhibitors of TbpA-hTf interactions as a possible therapy.

II. Results

A. Generation of MCV211 and MCV212

In an attempt to generate a mutated form of TbpA abrogated for its binding function, a mutational analysis was performed on various extracellular loops of TbpA. The mutational analysis by Cash *et al.* (281) on the α-helical finger of an extracellularly exposed loop from TbpA, found that the presence and amino acid sequence of the loop 3-helix is vital for TbpA function. Before this study, it was unclear which of the 11 surface-exposed loops played a significant role in transferrin binding, let alone metal piracy. The determination that loop 3 did significantly contribute to binding and removal of iron metal removal allowed for a more directed mutational analysis for follow-up attempts at the complete abrogation of TbpA function. However, a large surface area of TbpA interacts with the C-lobe of hTf, and Cash et al. used only single amino acid substitutions for the loop 3-helix mutational study. Therefore, to build off the previous research, we predicted that multiple amino acid substitutions of the loop 3- helix and other loops predicted to interact with hTf, could entirely abrogate ligand binding not detected with single mutations. Piliated FA19 cells were transformed with linearized pVCU191 or pVCU192 to generate MCV210 and MCV211, respectively. pVCU191 contains truncated *tbpB* and a mutated (D251A) full-length *tbpA*. Similarly, pVCU192 also has a truncated *tbpB* and a

mutated (D251A and K359R) full-length *tbpA* (**Figure. 4.2**). Western blots confirmed the expression of mutant TbpA from both MCV211 and MCV212 (**Figure 4.1**).

B. MCV210 and MCV211 exhibit reduced hTf binding

The TbpA D251A mutation in MCV210, was initially hypothesized to be the primary site of interaction between TbpA loop 2 and hTf, by Noinaj *et al.*, based on the co-crystal structure of *N. meningitidis* TbpA with hTf. The TbpA D251A mutation exhibited a significant reduction ($p < 0.05$=*) in HRP-Tf binding compared to WT FA6905. Significance was determined from 3 independent biological replicates performed in triplicate by a Students t-test. In the TbpA double mutant, MCV211 (D251A and K359R), there was no significant reduction in HRP-hTf binding when compared to WT FA6905 as determined by a Students t-tests (**Figure 4.3**). The introduction of the D251A loop 2 mutation or the D251A/K359R double mutation had a limited effect at reducing hTf interaction that was equivalent to the single L3H mutants previously tested via Cash *et al*.

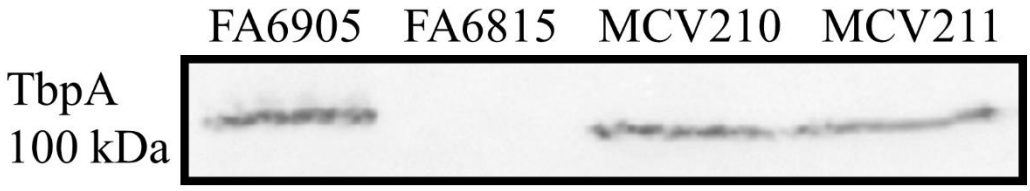

Figure 4.1 TbpA western blot of MCV210 and MCV211

Figure 4.1 TbpA western blot of MCV210 and MCV211

Strains FA6905 (WT), FA6815 (*tbpB*::Ω), MCV210 (D251A), and MCV211 (D251A/K359R) were grown in a chemically defined chelex treated media for 1 hour before back dilution and 4 hours of outgrowth. After 4 hours strains whole cell lysates were collected at 100,000 Klett Unit μL, suspended in 2x Laemmeli buffer and stored at -20 ^{o}C. Whole Cell lysates were subjected to SDS PAGE and transferred to nitrocellulose overnight at 28 mA. Blots were Ponceau stained to demonstrate equal loading of protein, and probed with 1:1000 α-TbpA 1^{o}, and then probed with 1:5000 goat α-rabbit IgG conjugated to horseradish peroxidase. The blot was developed with 4-chloro-1-naphthol/3,3'-diaminobenzidine tetrahydrochloride (CN/DAB substrate kit, ThermoFisher). FA6905, MCV210, and MCV211 all expressed TbpA while FA6815 did not.

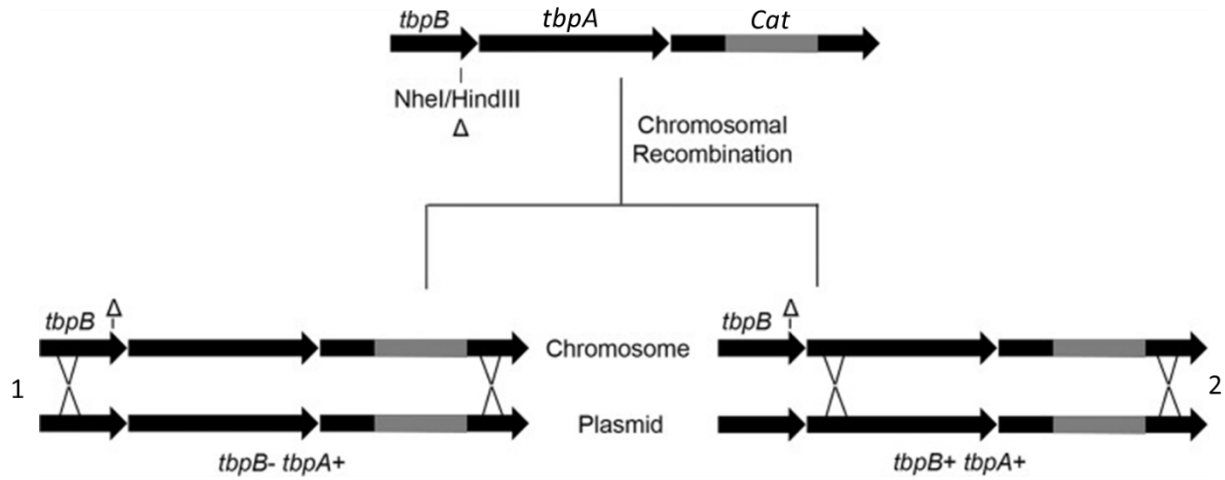

Figure 4.2 Schematic of pVCU191 and pVCU192 used to generate MCV210 and MCV211

Figure 4.2 Schematic of pVCU191 and pVCU192 used to generate MCV210 and MCV211.

The creation of MCV210 and MCV211 was achieved through the shuttle vector of pVCU191/192. These plasmids contain a deleted region of *tbpB* (*tbpB*Δ), full length *tbpA* and DNA sequence immediately downstream of the tbpA coding region which contains a mTn3*Cat* for a means of selection. pVCU191 possess a *tbpA* gene sequence with a D to A mutation at the 251 amino acid residues. pVCU192 possess a *tbpA* sequence that also contains this D251A mutation with an additional K to R mutation at the 359 amino acid residues. Gonococcal transformation with linearized pVCU191 and pVCU192 can result in two major possibilities. The first outcome is produced via a double cross over event in the *tbpB*Δ and in the downstream sequence of *tbpA* resulting in the genotype of the gonococcus being *tbpB*Δ *tbp*A+ Cm^r. The second possible outcome results from a double cross over event occurring in the *tbpA* sequence and in the downstream sequence of tbpA resulting in full length native *tbpB tbpA*+ Cm^r. Figure amended from Cash D.R. *et al.*

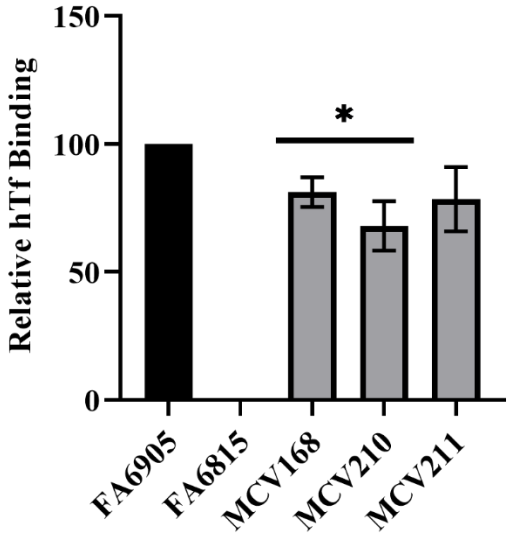

Figure 4.3 Mutant TbpA-hTf Binding ELISA

Figure 4.3 Mutant TbpA-hTf Binding ELISA

Iron starved gonococci grown on GCB media supplemented with 50 μM desferal were plated in 96 wells plates and probed with 12.5 nM HRP-hTf similar to previously-described methods and according to the manufacture's recommendations [Jackson Immunoresearch, (281)]. MCV210 contains an aspartic acid to alanine mutation at the 251 amino acid residue (D251). MCV212 has the same D251A mutation and an additional lysine to arginine change the 359 residue (K259R). MCV168 was used as an internal control as it had been previously published to have a significant reduction in HRP-hTf binding. MCV210 demonstrated an almost 30% reduction in HRP-hTf binding. MCV211 had an ~20% reduction in HRP-hTf binding, it was not statistically significant as determined by a Students t-test, error bars represent SEM.

C. Small molecule inhibitors of TbpA

Co-crystallization of *Neisseria meningitidis* TbpA in complex with hTf opened the doorway to a possible new means to treat gonococcal infections through the newly-gained insights into the intimate interactions of TbpA and hTf. Through a collaboration with Dr. Glen Kellogg at Virginia Commonwealth University, we virtually screened various databases of small molecules with the potential to disrupt the interaction between TbpA and hTf. Molecules were chosen based on their potential to fit in the binding pockets between TbpA and hTf (**Figure 4.4**). The original screen resulted in 15 candidates predicted to inhibit TbpA-hTf interaction at two potential sites. Putative compounds were chosen based off their hydrophobic interactions (HINT) scores, which are used to describe and quantify all possible biological interactions. HINT scores are multifactorial and include various biological interactions such as coulombic, hydrogen bonding, and hydrophobic interactions between the two molecules. The higher the HINT score, the more favorable the interaction between the small molecule and TbpA. The second screen of small molecules looked at the crystal structure of TbpA with the amino acid sequence of LbpA threaded through the crystal structure. This new analysis resulted in 16 compounds with predicted HINT scores suitable to disrupt the interaction of either TbpA or LbpA with their respective ligand and elucidated a potential third small molecule interaction site cross-reactive for LbpA and TbpA. The efficacy of these 16 compounds to disrupt hTf interaction with TbpA was determined via a modified transferrin binding ELISA.

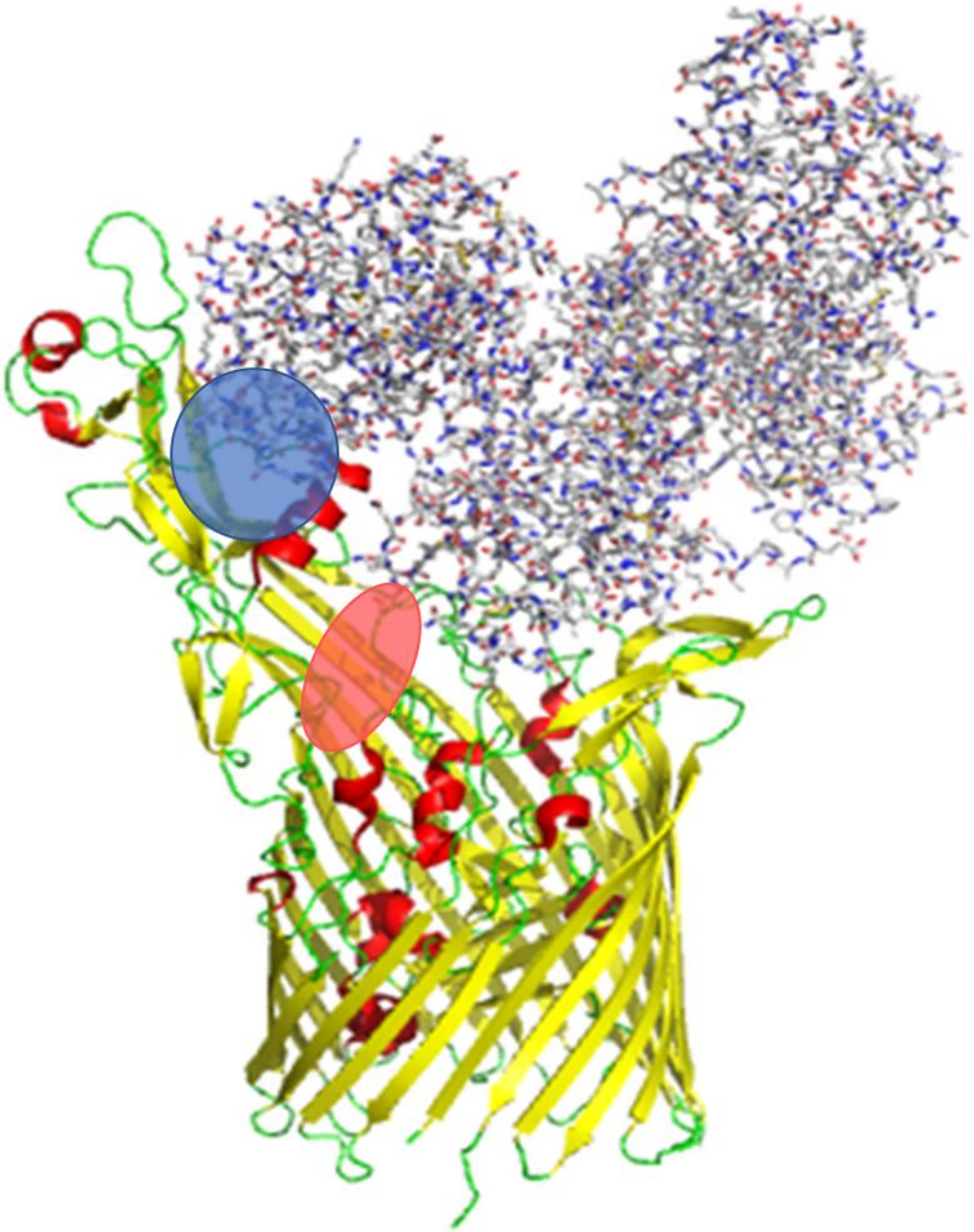

Figure 4.4 Location of small molecule binding site I and II

Figure 4.4 Location of binding site I and II.

TbpA co-crystal structure is show as a cartoon representation with colored secondary structures. α- helicies are shown in red, β-sheets are shown in in yellow and loop structures are shown as green. Transferrin is shown as a stick representation (grey) bound to TbpA with the putative small molecule binding site I shown in blue, site II shown in red. (PDB ID 3V8X)

In order to determine the sole contribution of our TbpA mutations, all of the strains used were in the FA6905 background (-*tbpB*) to eliminate TbpB binding to hTf. FA6905 was used as our positive control for hTf binding since it still expresses a function TbpA capable of interacting with hTf. FA6815 possess an Ω cassette in *tbpB* and prevents the expression of the transferrin acquisition system, and was used as a negative control due to the strains inability to interact with hTf. FA6905 and FA6185 were iron starved overnight by growth on GCB plates supplemented with 5 μM desferal before being resuspended and then added to a poly-L-lysine-coated 96-well microtiter plate. Compounds TL1 – TL16 were diluted in dimethyl sulfoxide (DMSO) to a stock concentration of 5 mM or 10 mM and added to the plate at 100 μM final concentration. DMSO served as a negative control illustrating a lack of hTf inhibition, while excess unlabeled Tf (comp) served as a positive control for the experiments. Compounds TL1- TL3 inhibited HRP-hTf binding to whole gonococci the least, showing no significant reduction compared to FA6905. Compounds TL5- TL7, TL-9 and TL-10 significantly decreased HRP-hTf interaction to whole gonococci, exhibiting a ~15-20% reduction in hTf binding to the surface of the gonococci (**Figure 4.5A**). Surprisingly, while TL11- TL16 had no significant decrease in HRP-hTf binding to the whole cells, these compounds slightly increased the interaction between hTf and the gonococcus (**Figure 4.5B**). These experiments could serve as a proof of principle for using small molecules to inhibit the receptor-ligand interaction could be a possible means of treatment for gonococcal infection.

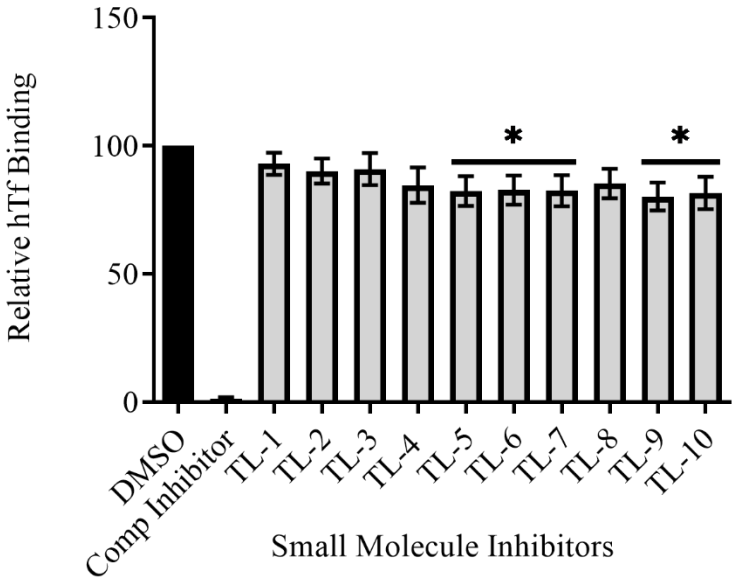

Figure 4.5A. Small Molecule inhibitors of TbpA ELISA with Compounds TL1- TL10.

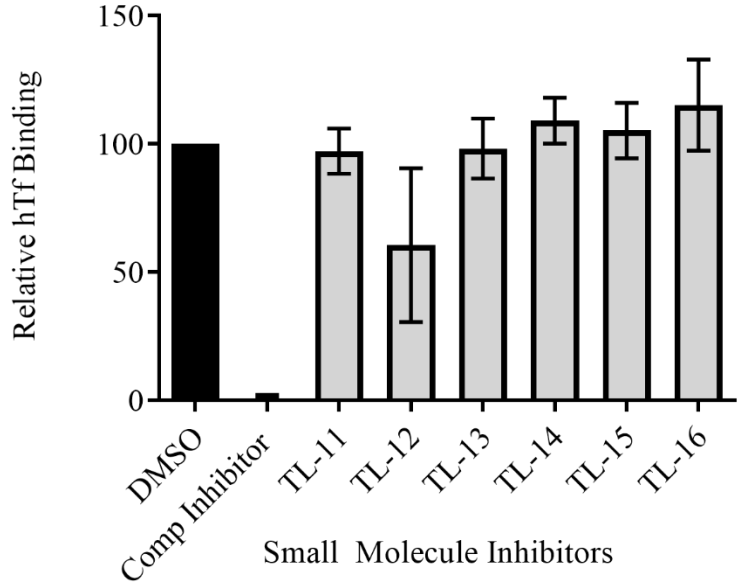

Figure 4.5B. Small Molecule inhibitors of TbpA ELISA with Compounds TL11- TL16.

Figure 4.5. Small molecule inhibitors of TbpA ELISA with compounds TL1- TL10.

A.) Gonococci grown on GCB Media containing 5 μM desferal, an Fe chelator, for Fe starvation overnight. Cells were resuspended to a final OD_{600} of 1.0 in PBS and transferred into a 96 well plate. Cells were incubated at room temp for 1h before cultures were blocked and treated with compounds TL1- TL10. Small molecules were in the plate for 1h and then probed with 12.5 nM HRP-hTf for 1h, washed, and developed with TMB ELISA substrate (ThermoFischer). Compounds TL-1, TL-2, and TL-3 had no significant reduction in binding when compared DMSO vehicle treated controls. TL-5 thru TL-7 and compound TL-9 and TL-10 demonstrated a significant reduction in HRP-hTf binding to the surface of the cell compared DMSO vehicle treated controls. TL-9 and TL-10 illustrated the greatest reduction in HRP-hTf binding, reducing HRP-Tf interaction by ~20%. **B.)** Compounds TL11- TL16 demonstrated little to no reduction in HRP-hTf binding; in fact, compounds TL14-TL16 increased HRP-hTf deposition to the surface of the cell. Significance was determined by a Students t-test ($p< 0.05$=*).

III. Discussion

The lack of a protective immune response and the continuing prevalence of gonococcal infections has made the need for an effective vaccine a top priority worldwide. Numerous attempts have been made, but due to the vast antigenic variation exhibited by *N. gonorrhoeae*, no efforts have been successful. However, the TonB-dependent transporters (TdTs) of the gonococcus have vaccine potential; specifically, TbpA is of particular interest, due to its lack of antigenic variation and conservation among all gonococcal isolates. A strain lacking TbpA expression, attenuated in its ability for colonization in a human male infection model, gives even more interest in further characterizing this protein for its vaccine potential (212). In 2012, the co-crystal structure of *N. meningitidis* TbpA with hTf was resolved. *N. gonorrhoeae* TbpA shares 95% sequence identity with *N. meningitidis,* and this co-crystal structure has furthered our understanding of how TbpA interacts with hTf (210).

Frandoloso et al. discovered that abrogation of interaction with host protein increases the immunogenicity of TbpB of *H. parasuis* in a piglet infection model. This led us to hypothesize that mutagenizing gonococcal TbpA so that it no longer interacted with human Tf would similarly result in increased immunogenicity and possibly protection. Cash *et al.* generated mutants in the L3H that were hypothesized to play a key role in iron removal from hTf. That study mutated the polar amino acids found within the L3H to characterize their role in hTf binding and iron removal. The mutation with the most significant phenotype was the terminal lysine residue of the L3H (K359), which resulted in a significant reduction in binding to hTf. The decrease in hTf binding also resulted in an overall reduction in iron accumulation into the cell. Therefore, we predicted that multiple mutations, one present in the L3H and a second mutation in loop 2, would further reduce or abrogate the ability of *N. gonorrhoeae* to bind to hTf.

The K259R mutation of the study by Cash *et al.* had been previously described as having reduced WT hTf binding capabilities (281), while the D251A mutation located in loop 2 was hypothesized to be 1 of the 87 amino acids that interact with hTf by Noinaj *et. al* (210).The D251A mutation alone resulted in almost 30% reduction from WT levels of binding to human transferrin. Interestingly, the combining mutations D251A/ K359R in MCV212 did not have an additive effect on the overall hTf binding. Despite the limited reduction in hTf association, only two mutations were analyzed in this study, which were selected based either on previously-observed ability to reduce hTf binding or a hypothesized interaction with hTf. The D251A and K359R mutations were also not present on the same loop or secondary structure, and their lack of proximity may have reduced their cumulative effects since such a large surface area of TbpA is thought to interact with hTf (210). The generation of multiple mutations across the surface of TbpA may further reduce the interaction of TbpA to hTf, as the surface area that is projected to interact between these two proteins is substantial. Further optimization of this study is currently ongoing with an extended survey of the function of the L3H via the introduction of prolines along the length of the helix. Loop 5 and loop 10 of TbpA have also demonstrated the ability to bind to hTf independent of the β-barrel and could also be coupled with the current mutations to abrogate hTf binding capabilities.

While the ultimate goal of our study is to continue investigating the potential of TbpA as a vaccine antigen, the dwindling rate of treatment options for gonococcal diseases poses a severe threat (286). Inhibition of the TbpA-HTf interaction can lead to a reduction in iron uptake into the cell. The co-crystal structure of the TbpA-hTf complex has allowed for the new innovative approaches at treating gonococcal disease including the use of small molecules that could interfere with the ability of TbpA to bind to hTf. An initial screening of TbpA's structure against

a small molecule database to identify compounds that inhibited the interaction between TbpA and hTf, identified 14 compounds with HINT scores suitable to potentially disrupt TbpA-hTf interactions with hTf. Testing these compounds for their ability to disrupt binding revealed that there was potential for these compounds to reduce TbpA-hTf interaction. A modest reduction was seen with two out of the 14 compounds tested; however, some of these compounds also increased the apparent interaction between hTf and TbpA (338). The second screening of small molecule database conducted utilized both the structure of TbpA and the amino acid sequence of LbpA threaded through the TbpA for its analysis. This screen found 16 putative compounds with HINT scores suitable to disrupt not just hTf interaction with TbpA but also hLf with LbpA. This approach may identify a new and innovative treatment option for gonococcal disease because a single compound could potentially disrupt two of the major iron uptake systems essential for the gonococcus survival in the host (212).

Similar to the previous small molecule study done by Dr. Devin Cash, ELISAs accessing the levels of transferrin binding identified a modest reduction with 7 out of the 16 compounds tested. The most significant inhibition of HRP-hTf binding was from compound TL-9 (Z45647810), which demonstrated about a 20% inhibition of hTf binding (Figure 1.4). These compounds still require optimizing. Modifying side chains of these compounds to mimic those naturally found in hTf may increase their solubility and stability, leading to a level of inhibition required for iron starvation. The level of hTf binding inhibition would need to be over 80-90% of WT TbpA to sufficiently starve the gonococci of iron if used independent of other antibiotics, but could have more modest inhibition if used as a therapy in conjunction with antibiotics (281). However, these compounds still require testing for the LbpA system to determine their reductive capabilities for the two systems. A putative third binding pocket of TbpA for these compounds

was also identified in the second screening of the small molecule databases, and a collection of the various site inhibitors needs to be tested as a multidrug inhibitor.

These compounds were selected for their ability to hydrophobically interact with TbpA. Therefore, attempts to modify these compounds chemical moieties to allow for increased ionic interactions may result in retention within these binding pockets, increasing the inhibitory nature of these molecules. Overall, the inhibition of the hTf-TbpA interaction from these small molecules was modest; however, continued testing of these compounds and others, alone and in combination, could reveal a new treatment for gonococcal disease if the right type and combination of compounds are determined.

A recent case of untreatable gonorrhea leaves a bleak outlook for the future of antibiotics as an effective treatment for the disease (339). Treatment with 250 mg intramuscular ceftriaxone and 1 g oral azithromycin, the currently recommended therapy, failed to treat and clear the infection. Characterization of this highly resistant strain revealed high levels of resistance to both azithromycin and ceftriaxone, the one of the first characterized strains of its kind. Our current study with the small molecule inhibitors of TbpA and LbpA shows potential for the inhibition of TbpA function through these compounds.

Our studies of TbpA have added to the knowledge and understanding of gonococcal TbpA function. The knowledge that a loop 2 mutation decreased ligand binding may allow for future mutagenesis across the various loops that, in conjunction with the current mutations, may abolish hTf binding by TbpA. An increased focus on the L3H mutations which disrupt the helical structure coupled with other loop mutations may ultimately provide a non-binding or non-functional form of TbpA which still retains its native conformation. These non-binding TbpA mutants potentially deployed with non-binding forms of TbpB, could represent an effective

vaccine with increased immunogenicity from the TbpB and cross-protection from TbpA. While a vaccine for the gonococcus is still the primary focus of our research, it cannot be forgotten that the continued prevalence of antibiotic resistance continues to increase every day. The recent case of an azithromycin and ceftriaxone resistant infection in the UK is likely the first of many to occur and, as such, new treatment potentials are desperately needed. The small molecule inhibitors of TbpA highlight a novel approach for the treatment of gonococcal disease and have the potential to be used as a stand-alone treatment or added with current therapies to treat resistant isolates.

Chapter 5: Analysis of the Putative Heme Exporter MacA in *N. gonorrhoeae*

I. Introduction

Hemoglobin, heme, and other heme-containing moieties constitute the majority of bio-available iron that pathogens use during infection, with more than 70% of the iron in humans contained within heme (158,292,340,341). Hemoglobin utilization and heme acquisition systems have evolved across a broad range of pathogenic bacteria to utilize this pool of bioavailable iron (327,340,342,343). Heme is a part of cytochromes in the electron transport chain (ETC), and the iron-bound to heme is used by enzymes as a cofactor and is an essential component of redox reactions and cellular respiration (159,340-342). Heme and heme-derivates are endogenously synthesized by *Neisseria* species, specifically *N. gonorrhoeae and N. meningitidis*, with the final step of synthesis being Fe^{3+} loading onto protoporphyrin IX (PPIX) catalyzed by HemH (344,345).

Excess heme has the potential for toxicity to both the pathogen and host due to production of reactive oxygen species (ROS) and to its lipophilic nature (346-348). Heme import and production in *N. gonorrhoeae* are tightly controlled. Endogenous heme synthesis is regulated by a negative feedback loop in *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella enterica*, involving PPIX-Fe inducing proteolytic degradation of the heme synthesis gene products , HemA, and HemH (344,349). *N. meningitidis* possess heme and hemoglobin uptake systems, HmrB and HpuAB respectively, and are both under Fur-regulation (144,220,223). Similarly, the gonococcal TonB-dependent transport system HpuAB is also tightly controlled by Fur and *N. gonorrhoeae* can utilize hemoglobin and hemoglobin-haptoglobin complexes through the HpuAB (228,350). The gonococcus has no known dedicated

heme importer, but *N. gonorrhoeae* is capable of using heme as a sole iron source in a TonB-independent manner (295).

Since heme has the potential for toxicity, *N. gonorrhoeae* has systems involved in detoxifying the protoporphyrin ring, or expelling excess heme from the cell. Bacterial efflux pumps maintain cellular homeostasis in toxic or otherwise hostile environments and can aid in the delivery of effectors to the extracellular tissue, which promotes pathogenicity. Pathogens that colonize and infect humans encounter host defenses and noxious compounds regularly and if maintained at high cellular levels results in bacterial cell death (351,352). Exporters of these potentially toxic compounds are energized in two ways. One way uses the proton motive force to stimulate the active transport of materials out of the cell, while the other uses intracellular energy in the form of ATP (353-355). Unlike most bacterial pathogens, there are few efflux pumps identified in the genome of *N. gonorrhoeae*, with MacA annotated as a macrolide exporter and a putative transporter for heme export (353,356). MacA interacts with TolC found in the outer-membrane of *E. coli* and *N. gonorrhoeae*. The MacA-TolC channel is energized via the ATPase, MacB, creating a tripartite efflux pump initially identified to transport macrolide antibiotics out of the cell (353,357). *E. coli* MacAB has been shown to export heme from the cell and deliver enterotoxin II to the extracellular environment aiding in *E. coli* colonization and pathogenesis (352). Targeting a potential pathway for heme extrusion could be a possible new chemotherapeutic treatment for gonococcal disease or disseminated infection given the dwindling options for treating gonococcal disease. Through a collaboration with Dr. William Shafer at Emory University, we sought to determine if the efflux system, MacAB, was crucial in heme export from *N. gonorrhoeae*.

II. Results

Strains FA19 (WT), macA::kan (MacA KO), and two MacA complementation strains, pGCC3+::*macA*^{C} and pGCC4+::*macA*^{C}, were provided to us by Dr. Shafer. pGCC3+::*macA*^{C} and pGCC4+::*macA*^{C} inserts MacA at an ectopic site within the chromosome and contains two promoter operator sequences allowing for IPTG control of gene transcription. Strains were grown overnight on GCB media supplemented with 50 μM desferal for overnight iron starvation before inoculation into CDM. Cells were grown for around one hour before back diluting in a CDM mixture containing 2.5 μM apo-bTf as an iron chelator and plating into a 96-well plate containing various dilutions of heme (5 μM, 10 μM, 15 μM, and 25 μM heme), 30% saturated hTf, or 30% saturated bTf as iron sources, and 1 mM IPTG. Strains were grown for six hours with hourly time points taken (**Figure 5.1-Figure 5.4**).

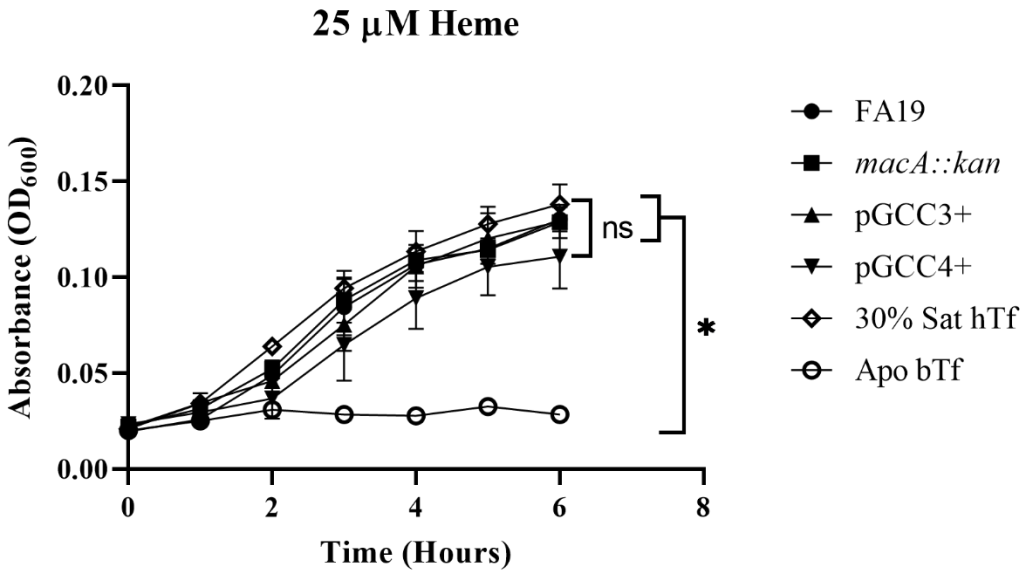

Figure 5.1 Heme-dependent growth of *N. gonorrhoeae* with 25 µM heme

Figure 5.1 Heme-dependent growth of *N. gonorrhoeae* with 25 μM heme

Strains FA19 (WT) , *macA::kan (*MacA KO), pGCC3+ *macA*^{C}, and pGCC4+ *macA*^{C} were grown overnight on GCB supplemented with 5 μM desferal before inoculation of a trace metal free flask. Strains were grown until doubling before back dilution with CDM containing 2.5 μM apo-bTf for free iron chelation. Back diluted strains were plated in a 96-well plate with 25 μM heme, 2.5 μM 30% saturated hTf as a positive control, or no iron source as a negative control. Bacterial cultures were incubated at 37^{o}C with 5% CO_2 for 6 hours with hourly OD readings. No significant difference seen between strains FA19, MacA KO, or either MacA complementation strains. Every strain, with the exception of pGCC4+, was significantly different from FA19 grown with apo-bTf ($p< 0.05$= *). Statistical analysis performed via a two-way ANOVA with Tukey's multiple comparisons *post-hoc* test. Error bars represent standard error of the mean (SEM).

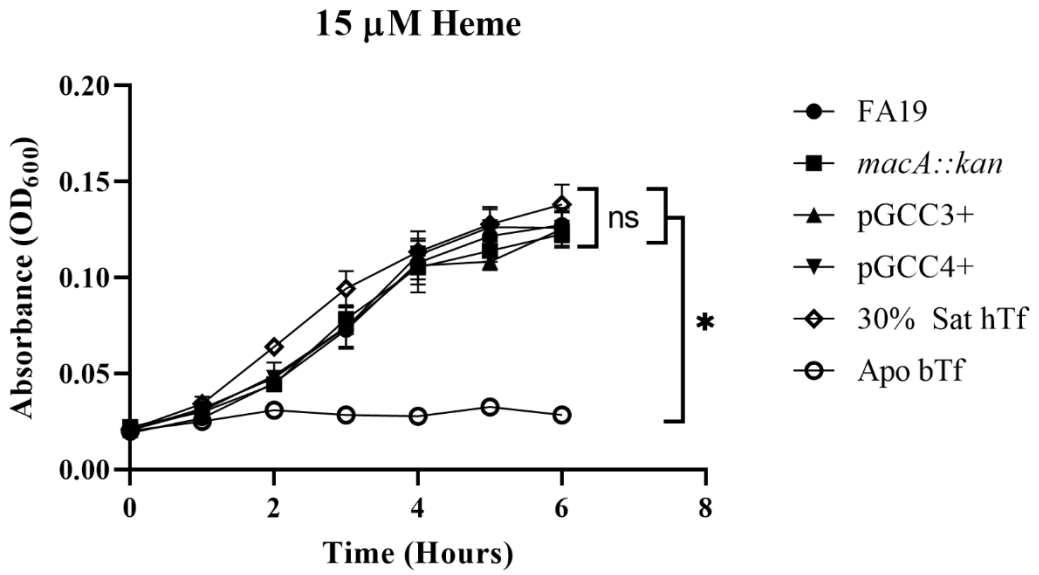

Figure 5.2 Heme-dependent growth of *N. gonorrhoeae* with 15 µM heme

Figure 5.2 Heme-dependent growth of *N. gonorrhoeae* with 15 μM heme

Bacterial strains were grown, back diluted, and plated as in figure 2.1 and grown in the presence of 15 μM heme. No significant difference seen between stains FA19, MacA KO, or either MacA complementation strain. Every strain was significantly different from FA19 grown with apo-bTf ($p < 0.05$= *). Statistical analysis performed via a two-way ANOVA with Tukey's multiple comparisons *post-hoc* test. Error bars represent SEM.

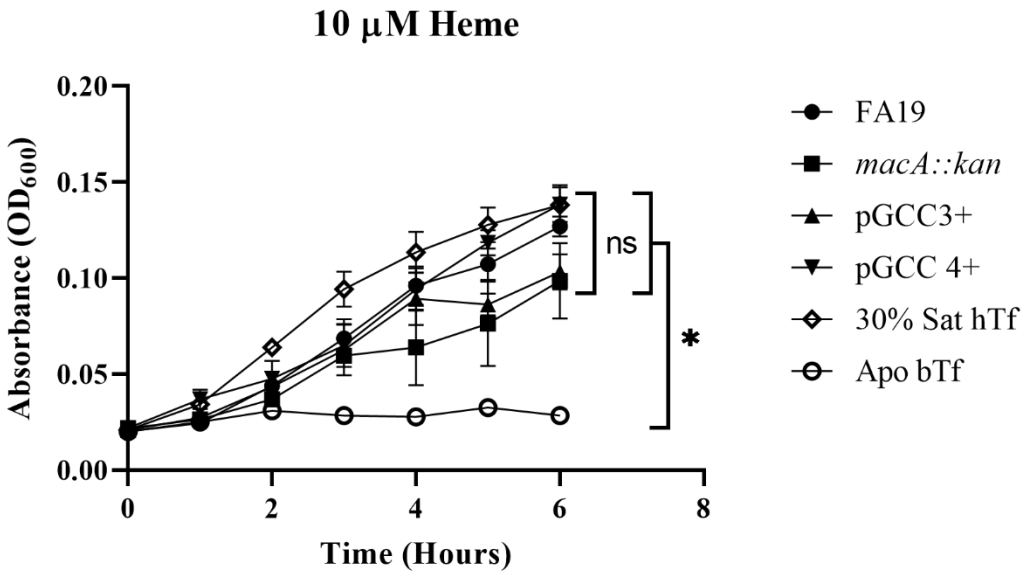

Figure 5.3 Heme-dependent growth of *N. gonorrhoeae* with 10 µM heme

Figure 5.3 Heme dependent growth of *N. gonorrhoeae* with 10 μM heme.

Bacterial strains were grown, back diluted, and plated as in figure 2.1 and grown in the presence of 10 μM heme. No significant difference seen between stains FA19, MacA KO, or either MacA complementation strain. Every strain was significantly different from FA19 grown with ap-bTf ($p < 0.05$= *). Statistical analysis performed via a two-way ANOVA with Tukey's multiple comparisons *post-hoc* test. Error bars represent SEM.

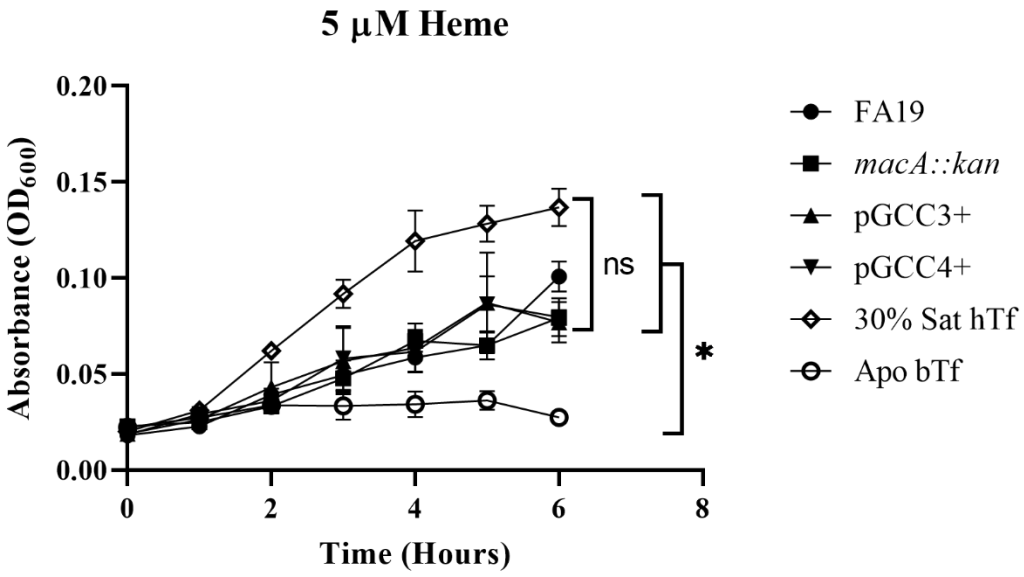

Figure 5.4 Heme-dependent growth of N. gonorrhoeae with 5 μM heme

Figure 5.4. Heme-dependent growth of *N. gonorrhoeae* with 5 μM heme.

Bacterial strains were grown, back diluted, and plated as in figure 5.1 and grown in the presence of 5 μM heme. Every strain was significantly different from FA19 grown with apo-bTf ($p< 0.05$= *). Statistical analysis performed via a two-way ANOVA with Tukey's multiple comparisons *post-hoc* test. Error bars represent SEM.

We hypothesized that the MacA KO strain would be defective for growth as compared to strains grown in 30% saturated hTf. However, the MacA KO showed no reduction in growth for all the heme dilutions tested compared to the positive control. The MacA KO mutant consistently grew at the same rate and to a similar final OD_{600} as the WT and complemented strains. The 5 μM heme concentration did exhibit slightly reduced growth for all strains when compared to the positive control, but is possibly an artifact of the reduced iron availability to the iron starved gonococci.

Ferrochetelase, or *hemH*, is responsible for the catalytic addition of Fe^{3+} to heme and heme derivatives within cells (345). The lack of any growth defect in the MacA KO mutant led us to question if the ability to load endogenous heme by *hemH* was mitigating the toxicity of the heme concentrations used, and thus we sought to generate a double mutant of MacA KO and *hemH::Cat*. Unfortunately, transformants were not able to be recovered, which may have been due to the essential roles that both *macA,* and *hemH* have together for the gonococcus. Taken together these heme growth assays strongly suggest that MacA is not an exporter of intracellular heme for *N. gonorrhoeae*.

I. Discussion

Heme plays a crucial role in survival, colonization, and pathogenesis for many bacterial species, but can also pose a threat to bacterial cells incapable of regulating its import, export, or production (329,340). When heme is present at high concentrations in a bacterial cell, it can generate harmful ROS and can insert itself into membranes due to its lipophilic nature (346,348). Recently, studies have identified a heme efflux pump in *E. coli*, MacAB (344). MacAB was initially annotated as an antibiotic efflux pump for macrolide antibiotics in several pathogenic species (353,358,359). MacA forms a periplasmic channel interacting with TolC to generate a

channel spanning from the inner-membrane to the outer membrane of the bacterial cell. MacB has ATPase function and energizes the MacA-TolC complex to expel macrolides and heme out of *E. coli* (344,353). Pathogenic *Neisseria*, like many other pathogens, can directly import heme via outer membrane receptors. *N. gonorrhoeae* has a hemoglobin transporter, HpuAB, which binds to hemoglobin and transports heme into the cell (360-362). Growth assays of an isogenic mutant with an inactivated MacA were used to test if the MacAB system of *N. gonorrhoeae* plays a similar role to that of *E. coli*.

A deficiency in the growth of the MacA KO mutant strain would have been evidence for a role that MacA is an exporter of heme for the gonococcus. The dilutions of heme were chosen to show toxicity at lower dilutions, given the documented inhibitory concentration of around 25 μM heme in *N. meningitidis* (363). Over the six hours of growth in the presence of either 25 μM, 15 μM, 10 μM, or 5 μM heme, the MacA KO strain demonstrated no significant reduction in growth under the conditions tested. The MacA KO mutant grew as well as the positive control strain for all dilutions except for 5 μM heme. Stains grown in 5 μM heme as the sole source of iron had a lower final OD_{600} and slower growth compared to the positive control grown in 2.5 μM hTf. All of the strains, including the WT, showed reduced growth in 5 μM heme, which may be due to the lack of iron availability in the iron starved cells. Since heme requires proteolytic degradation for pathogens to access the Fe^{3+}, there possibly was not enough heme to allow for maximal growth. Further, *hemO*, which encodes heme oxygenase, is responsible for the proteolytic degradation of heme allowing for iron access and detoxification of the protophoryin ring, was still present in the MacA KO strain (344,364). The degradation of imported heme by HemO may have reduced the toxic accumulation occurring from the lack of heme export through

MacA; therefore, future experiments should be conducted with a double *hemO macA* mutant strains to determine if MacA is responsible for *in vitro* efflux of heme in *N. gonorrhoeae*.

Chapter 6 Perspectives and Future Directions

The rate of gonococcal infections has been steadily increasing worldwide. In the United States alone, there were over 500,000 confirmed cases with an estimated 800,000 cases of gonococcal infection in 2018, as reported by the CDC (45). The increase in gonococcal infections causes an increased financial burden on the health care of infected individuals (365). Even more concerning is the growing number of strains exhibiting high levels of resistance to clinically useful antibiotics [(60,286), **Table 3**]. The recent isolation of a gonococcal strain in the United Kingdom, which exhibited extensive resistance to the current dual therapy, ceftriaxone, and azithromycin, means that the potential for untreatable gonorrhea is now a reality (339). The growing concern of untreatable gonorrhea can be seen in the recent characterization of *N. gonorrhoeae* as an urgent threat by the CDC.

To combat the growing risk of antibiotic-resistant gonococcal isolates, continued investment in the development of new antibiotics is crucial. Several promising candidates are in the developmental pipeline and are at various stages of development, with solithromycin being the furthest along. Solithromycin (CEM-101) is a fluoroketolide that inhibits the 50S ribosomal subunit. Solithromycin was found to have a MIC range of 0.001-32 μg/ mL against over 200 gonococcal isolates, which is lower than other macrolides, like the currently prescribed azithromycin (366). However, some issues have arisen during its on-going phase III clinical trial testing the effectiveness of solithromycin as a therapeutic for gonococcal infection (367). A study comparing solithromycin to the current recommended dual therapy, ceftriaxone, and azithromycin, found that after 7 days, solithromycin was inferior in treating uncomplicated gonococcal urethral infection (367). Despite the setback in the development of solithromycin as a

new treatment for *N. gonorrhoeae*, the phase III trial is still on-going and has, so far, the best potential as a new treatment for uncomplicated gonococcal infections.

Table 3 Antibiotics and Gonococcal Resistance Mechanisms

Drug Class	**Resistance Mechanism (371)**
Penicillin	Alteration of penicillin-binding proteins (PBPs) Plasmid encoded production of penicillinases Alteration of porins that limit penicillin influx Changes in Mtr efflux pump expression
Cephalosporins	Mutations in PBPs Alteration of porins limiting cephalosporin influx Changes in Mtr expression
Macrolides	Mutations in the 23s ribosomal subunit Alteration in efflux pump expression
Fluoroquinolones	Mutations in topoisomerases II and IV
Tetracyclines	Expression of TetM, protection protein Alterations of the target structure Alterations in porin that limit tetracycline influx Changes in efflux pump expression

Another of the promising new drug candidates is the new DNA gyrase inhibitor Zoliflodacin (AZD0914), which has completed phase II trials and begun phase III. Zoliflodacin is a spiropryimidinetrione, which is a novel derivative of a quinolone drug and was found to have high *in vitro* activity against over 250 gonococcal isolates, including extensively drug-resistant strains (368). In the phase II clinical trial, patients suffering from uncomplicated urogenital, rectal, and pharyngeal infections were given a 3 g single-dosage of Zoliflodacin. The 3 g single-dosage was able to cure 100% of rectal infections and 96% of urogenital infections; however, Zoliflodacin was less able to cure patients with pharyngeal infections (82%) compared to patients who were prescribed ceftriaxone (100%) (369). Even though Zoliflodacin was less able to treat pharyngeal infections it still has potential to treat rectal and urogenital gonococcal infections and, phase III clinical trials are now underway. While the current drugs in clinical trials are the closest to being effective treatments for gonococcal infections, investing more research into the discovery of new antibiotics is still of paramount importance. The newly discovered antibiotic darobactin was found to have both *in vitro* and *in vivo* efficacy against Gram-negative pathogens (370). Darobactin acts against the outer-membrane protein BamA, which effectively inactivates the Bam complex and presents a novel mechanism of action (370). There is much work left to be done with these new drugs to fully determine their efficacy as a treatment option for gonococcal infection, particularly in more complex cases of infection, but they are promising as the potential for untreatable gonococcal disease becomes a reality.

Including the drugs in clinical trials, there are several other drugs currently being studied with promising *in vitro* data. However, these compounds belong to drug classes that the gonococcus has developed some resistance too (371). In order to prevent gonococcal infections, the development of a preventative gonococcal vaccine must occur. This study investigates the potential of a TonB-dependent transporter as a target for novel drug development. Specifically, we sought to target the Tbp system of the gonococcus, given its importance to infection (212). We further investigated the potential of another TdT as a vaccine candidate by investigating the structural and functional relationship to the Cp-Zn acquisition system, TdfH, for the potential addition to a multi-component gonococcal vaccine. The small molecule inhibition of hTf-binding to TbpA, while minimal, shows promise. These small molecules could be modified to better inhibit the interaction between TbpA and hTf, which could lead to a promising treatment option. The ability of our first-generation small molecules, which have undergone no additional modifications, to inhibit hTf binding to TbpA even minimally is a proof-of-concept that these small molecules are potentially able to be a new therapeutic. However, extensive work will need to be done to tailor these molecules further to inhibit binding to between TbpA and hTf to the degree needed to limit the growth of the gonococcus.

A growing body of evidence shows that presenting host binding proteins as "naked" antigens, which are unbound by ligand, elicits a better immune response and produce antibodies that are cross-protective and bactericidal compared to their native forms (326,372,373). Our mutational analysis of TbpA using mutations present in the L3H and in loop two was a follow up of the TbpA analysis by Cash *et al*. (281), which found that the L3H was important to binding and use of hTf. The study also determined that single residue charge changes of the L3H amino acids were not sufficient or capable of reducing hTf binding enough to inhibit the growth of *N.*

gonorrhoeae. Our study introduced an additional mutation in loop two of TbpA, which was predicted to have an intimate interaction with hTf (210). Despite the predicted intimate interaction of the D251 residue, we were only able to measure at best a 30% reduction of hTf binding, even when added to the K359R L3H mutation (**Figure 4.3**). Other mutational studies are currently underway that utilize proline mutations meant to disrupt the L3H but maintain the overall structure of TbpA in order to generate the best possible candidate for the inclusion into a gonococcal vaccine.

The co-crystal structure of TbpA and hTf has informed much of our experimental design in the mutational and small molecule experiments (210). Studies elucidating the crystal structure of the gonococcal CP binding and Zn piracy protein, TdfH, is currently underway by our co-author Dr. Nicholas Noinaj but is still unresolved. *N. gonorrhoeae* upregulates a Th17 response to drive neutrophils to its site of infection (57). Interestingly, CP is one of the most abundant cytosolic and granular proteins of a neutrophil (297). TdfH also allows the gonococcus to survive when trapped inside of NETs by being able to overcome the high levels of metal sequestration of the NET environment through its ability to hijack Zn from CP (231). Thus, the CP-gonococcal interaction provides an explanation, at least in part, as a reason that the gonococcus drives neutrophils to the site of infection, which "delivers" an extremely abundant Zn source that the gonococcus can hijack (231).

The combined lack of a TdfH crystal structure and the critical role TdfH may have for gonococcal survival during infection prompted us to evaluate the structural and functional relationship of TdfH and CP. Our studies determined that mouse CP was unable to support the growth of the gonococcus when it was supplied as the sole Zn source. Furthermore, like most of the other gonococcal TdTs, the human form of CP was able to interact with the gonococcus

while the mouse form of CP could not, and that interaction was determined to be high affinity [(4.0 nM), **Figure 3.5**]. CP is unique among S100 proteins due to its preference for S100A8 and S100A9 to heterodimerize (297). This heterodimerization creates two distinct transition metal-binding sites, with one metal-binding site being canonical to those found among other S100 proteins and the second being unique to CP (194,204). Our study determined that Zn piracy occurs from the unique, non-canonical binding site of CP as gonococci grown with a mutant form of CP where that site was unable to bind metals was deficient for growth (**Figure 3.7**). The non-canonical transition metal binding site is also capable of sequestering Mn and several other transition metals with high affinity and could thus potentially mean that TdfH is a transporter of metals aside from Zn (203,204,301). However, preliminary data shows that Mn plays an insignificant role in the overall growth of the gonococcus, and thus, more investigations will be needed to determine if TdfH is only a Zn transporter or if it can import other transition metals that may be bound to CP. In the absence of a crystal structure for TdfH, we generated a homology model and predicted loops and beta-strands presented as a 3D structure (**Figure 3.9**). This homology model gives us a starting point moving forward of where extracellularly available loops that may play an integral role in the interaction with CP are, and may allow further structure-function studies aimed at parsing out the loops that are critical to TdfH-CP interaction. The knowledge that metal piracy is occurring from the non-canonical metal-binding site and our generation of a homology model can also inform future mutagenesis experiments, similar to those that have been performed on the Tbp system, which could result in the inhibition of ligand binding and consequent Zn piracy (281). Since TdfH is primarily produced by the pathogenic *Neisseria,* a non-ligand binding version of TdfH could be included in a cocktail vaccine and

would leave the commensal *Neisseria* population intact while being cross-reactive for most of the strains of *N. gonorrhoeae* and *N. meningitidis*.

In our final project of this study, we moved away from the targeting of TonB-dependent transporters and focused on a known efflux pump of the gonococcus. MacA is a macrolide exporter of *N. gonorrhoeae*, and it was initially found to export macrolide antibiotics out of the cell. Recent reports have found that MacA also contributes to limiting heme toxicity by exporting heme out of the cell in the Gram-negative pathogen, *E. coli* (344). Heme plays an essential role in energy acquisition and is a component of cytochromes of the ETC (342). However, heme is a double edge sword, and maintaining an appropriate concentration inside the cell mitigates the harmful toxicity that high concentrations of heme have (160). Our preliminary data of a MacA deficient gonococcal strain, which were grown on increasing concentrations of heme, found that MacA played no role in exporting heme out of the cell (**Figure 5.1-5.4**). However, further studies into MacA will be needed to determine if this gonococcal exporter has a role in heme homeostasis for *N. gonorrhoeae*

Taken together, these studies provide further structure and functional knowledge of the TdTs of *N. gonorrhoeae* and provides more evidence of the potential that targeting TdTs have as a therapeutic or vaccine antigen to prevent future gonococcal disease. Understanding the intimate protein-protein interactions between TdTs and their respective ligands will allow a more detailed analysis of protein structures that are critical for function. Gonococcal TdTs remain highly valued for their potential use as vaccine antigens due to their lack of high-frequency phase or antigenic variation, conservation across strains, and their importance to the virulence of the gonococcus. These findings have the potential to be the building blocks for future studies aimed

at the development of protective gonococcal vaccine and therapeutics for the treatment of gonococcal disease as infections continue to rise and may soon be untreatable.

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Vita

Michael Timothy Kammerman was born May 19th 1992 in Putnam, CT. He graduated from Mills E. Godwin Highschool, Richmond, VA in 2010. In 2015, Michael received his Bachelors of Science in Biology from Virginia Commonwealth University, Richmond, VA. He matriculated in Virginia Commonwealth University School of Medicine Biomedical Sciences Doctoral Portal in 2015 and into the Department of Microbiology and Immunology in the fall 2016. His accomplishments and publications are listed below.

Presentations

Poster

Neisseria gonorrhoeae TdfH Exhibits Species Specificity Ligand Binding for Human Calprotectin. **MT Kammerman**, CN. Cornelissen. American Society for Microbiology Microbe 2017 Meeting, New Orleans, LA, June 2017.

Affinity of the interaction of *N. gonorrhoeae* TdfH for its ligand Human Calprotectin. **MT Kammerman**, WJ Chazin, N Noinaj, CN Cornelissen. American Society for Microbiology Microbe 2018 Meeting, Atlanta, GA June 2018.

Oral

Characterization of the TdfH-calprotectin interaction that enables *Neisseria gonorrhoeae* to overcome Zn-specific nutritional immunity. **MT Kammerman**, A Berra, N Noinaj, W Chazin, CN Cornelissen. XXIst International Pathogenic Neisseria Conference, Pacific Grove, CA, September 2018.

Publications

Kammerman MT, Bera A, Wu R, Harrison SA, Maxwell CN, Lundquist K, Noinaj N, Chazin WJ, Cornelissen CN. 2020. Molecular insight into TdfH-mediated zinc piracy from human calprotectin by *Neisseria gonorrhoeae*. mBio11:e00949-20.https://doi.org/10.1128/ mBio.00949-20.