Functional and Immune Characterization of the Gonococcal Zinc Importer TdfJ

Stavros Maurakis

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Functional and Immune Characterization of the Gonococcal Zinc Importer TdfJ

by

Stavros Andrew Maurakis

Under the Direction of Cynthia Nau Cornelissen, Ph.D.

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the Institute for Biomedical Sciences

Georgia State University

2022
Neisseria gonorrhoeae causes the eponymous sexually transmitted infection gonorrhea, a global disease that afflicts millions of individuals worldwide per year. To date, no effective vaccine to prevent gonococcal infection has been produced, and the pathogen rapidly acquires and maintains mechanisms for antimicrobial drug resistance. Further, natural infection by N. gonorrhoeae elicits no protective immune response, making reinfection a common outcome. During infection, the gonococcus employs an arsenal of TonB-dependent outer-membrane transporters (TdTs) which facilitate the acquisition of essential nutrients such as iron and zinc from the human host, which itself goes to great lengths to restrict these metals’ availability. The TdTs are well conserved, surface accessible, and play critical roles in gonococcal pathogenesis, making them promising targets for therapeutic and/or vaccination efforts. One TdT, called TdfJ, allows the gonococcus to acquire zinc from S100A7, an innate immunity protein that typically suppresses bacterial growth via zinc sequestration. TdfJ contains an α-helix motif in its extracellular loop 3 (L3H), a conserved structure which has been shown to participate in the binding of and subsequent iron extraction from transferrin by another TdT, TbpA. In this report, we generated a series of mutations in the TdfJ L3H and assessed their impacts on S100A7 binding and utilization. We found that proline insertions at residues K261 and R262 fully abrogated the interaction between the two proteins, highlighting the importance of this motif in metal acquisition. We went on to explore the functionality of TdfJ as a vaccine antigen via two approaches. In one approach, we grafted extracellular loop sections of TdfJ onto a soluble lipoprotein scaffold and immunized mice, whose post-immune sera and vaginal secretions were screened for TdfJ-reactive antibodies. In another, we formulated recombinant TdfJ with
multiple adjuvants to assess the duration of gonococcal colonization in mice and whether adjuvant type affected efficacy. These two approaches demonstrated that TdfJ as a vaccine antigen is protective in a mouse model of infection, and that antibodies generated during upon vaccination may inhibit gonococcal ability to utilize S100A7. Taken together, these data may direct the path towards future prevention of gonococcal infections.

INDEX WORDS: Neisseria gonorrhoeae, gonorrhea, sexually-transmitted infections, vaccine
Functional and Immune Characterization of the Gonococcal Zinc Importer TdfJ

by

Stavros Andrew Maurakis

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Timothy Denning, PhD
Zehava Eichenbaum, PhD

Electronic Version Approved:

Office of Academic Assistance – Graduate Programs
Institute for Biomedical Sciences
Georgia State University
July, 2022
DEDICATION

This work is dedicated to all those who inspired my academic journey. To my parents, Tim and Jo Ann, thank you for instilling in me the drive to always set and pursue new goals, the desire to ask questions and never miss an opportunity to learn, and the responsibility to make a positive impact. And thank you for your unwavering support, patience, and love you’ve always shown. To Catherine and Michael, thank you for always encouraging me and leading by example, and for always being there when I’ve needed the kind of friendship only a sibling can give. To my wife, Sara, thank you for your unwavering companionship, your endless capacity for patience and empathy, and your immeasurable ability to bring out the best in people. You inspire me daily. To my friends, though you are too numerous to name, thank you for always being there to distract me from life’s stresses and make memories we’ll always cherish.
ACKNOWLEDGEMENTS

First, I would like to thank my mentor, Dr. Cynthia Cornelissen, for her support, guidance, and encouragement during my graduate studies. She has helped me to grow not only as a young scientist, but as a human as well. She has always offered insightful discussion on not only scientific matters, but matters of life as well. I am forever grateful to have learned from someone so enthusiastic about developing the next generation of scientists, and I will look to her as a role model for the rest of my scientific career. I would also like to thank the members of my advisory committee, Dr. Timothy Denning and Dr. Zehava Eichenbaum. Their guidance and perspectives have been instrumental in the completion of the research described herein, and they have significantly increased the breadth with which I think on these projects. I also owe many thanks to our collaborators, including but not limited to the laboratories of Drs. Walter Chazin, Nicholas Noinaj, Alison Criss, Scott Gray-Owen, Trevor Moraes, and Tony Schryvers. The completion of this project simply would not have occurred without each of these. Similarly, I would not be here if not for the former and current members of the Cornelissen lab, all of whom have given me support, ideas, friendship, and a healthy dose of laughter over the years. Finally, I would like to thank everyone involved in graduate education at Georgia State University. They have been an invaluable resource and have always been quick to answer whatever questions or guide me in whichever direction was needed during my time here, and I applaud their sincere dedication to education.
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LIST OF ABBREVIATIONS

>  greater than
<  less than
\%  percent
\sim  approximately
\degree\text{C}  degrees Celsius
\alpha  alpha
\beta  beta
\Delta  deletion
\Delta L3H  loop 3 helix deletion
\mu g  microgram
\mu L  microliter
\mu M  micromolar
2D  two-dimensional
3D  three-dimensional
A_{450}  absorbance at 450 nanometers
ABC  ATP-binding cassette
ADCC  antibody-dependent cell-mediated cytotoxicity
ANOVA  analysis of variance
AP  alkaline phosphatase
apo-  empty/no metal bound
ATP  adenosine triphosphate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl-phosphate</td>
</tr>
<tr>
<td>BMDC</td>
<td>bone marrow-derived dendritic cell</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C-</td>
<td>carboxy</td>
</tr>
<tr>
<td>C1q</td>
<td>complement component C1q</td>
</tr>
<tr>
<td>C3</td>
<td>complement component C3</td>
</tr>
<tr>
<td>C4bp</td>
<td>complement component C4b binding protein</td>
</tr>
<tr>
<td>Ca/Ca$^{2+}$</td>
<td>calcium</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CDM</td>
<td>Chelex-treated chemically-defined medium</td>
</tr>
<tr>
<td>CEACAM</td>
<td>carcinoembryonic antigen-related cell adhesion molecules</td>
</tr>
<tr>
<td>CFA</td>
<td>complete Freund’s adjuvant</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit(s)</td>
</tr>
<tr>
<td>CMP-NANA</td>
<td>cytidine-5’-monophospho-N-acetylneuraminic acid</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CN</td>
<td>4-chloro-1-napthol</td>
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<tr>
<td>Co-NTA</td>
<td>cobalt-activated nitriltriacetic acid</td>
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<td>CO$_2$</td>
<td>carbon dioxide</td>
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<td>CTA/CTB</td>
<td>Cholera toxin subunit A/B</td>
</tr>
<tr>
<td>Cu/Cu$^{2+}$</td>
<td>copper</td>
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CV: column volume
DAB: 3,3'Diaminobenzidine
dH₂O: deionized water
DNA: deoxyribonucleic acid
DTT: dithiothreitol
EC₅₀: 50% binding saturation
ECL: enhanced chemiluminescence
EDTA: ethylenediaminetetraacetic acid
ELISA: enzyme-linked immunosorbent assay
Fe: iron
Fe²⁺: ferrous iron
Fe³⁺: ferric iron
FeCl₂: ferrous chloride
Fe(NO₃)₃: ferric nitrate
fur: ferric uptake regulator
g: gram
GCB: gonococcal broth
GSK: GlaxoSmithKline
h: hours
H₂SO₄: sulfuric acid
HA: hemagglutinin
Hb: hemoglobin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>Hp</td>
<td>haptoglobin</td>
</tr>
<tr>
<td>HPV</td>
<td>human papillomavirus</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>inductively-coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>IgA</td>
<td>immunoglobulin A</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IP</td>
<td>intraperitoneal/intraperitoneally</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>K&lt;sub&gt;D&lt;/sub&gt;</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>KLH</td>
<td>keyhole limpet hemocyanin</td>
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<tr>
<td>L3H</td>
<td>loop 3 helix</td>
</tr>
<tr>
<td>LAMP-1</td>
<td>lysosomal associated membrane protein 1</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
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<td>LOS</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>M</td>
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</table>
mAb  monoclonal antibody
MAPK  mitogen-activated protein kinase
mg  milligram
MHC  major histocompatibility complex
min  minute
mL  milliliter
mM  millimolar
Mn/Mn⁡²⁺  manganese
MPL  monophosphoryl lipid A
MSM  men who have sex with men
MWCO  molecular weight cutoff
N-  amino
NaCl  sodium chloride
NaHCO₃  sodium bicarbonate
NBT  nitro blue tetrazolium
NCBI  National Center for Biotechnology Information
NET  neutrophil extracellular trap
NF-κB  nuclear factor kappa-light-chain enhancer of activated B-cells
NiCl₂  nickel chloride
Ni-NTA  nickel-activated nitrilotriacetic acid
NK  natural killer
NLRP3  NOD-, LRR- and pyrin domain-containing protein 3
<table>
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<tbody>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>nM</td>
<td>nanomolar</td>
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<tr>
<td>NTA</td>
<td>nitrilotriacetic acid</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>optical density read at 600 nanometers</td>
</tr>
<tr>
<td>OMV</td>
<td>outer-membrane vesicle</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDB</td>
<td>protein data bank</td>
</tr>
<tr>
<td>PEA</td>
<td>phosphoethanolamine</td>
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<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
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<td>poly-C</td>
<td>poly-cytosine</td>
</tr>
<tr>
<td>poly-G</td>
<td>poly-guanine</td>
</tr>
<tr>
<td>PSI</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>RAGE</td>
<td>receptor for advanced glycation end products</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPM</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RU</td>
<td>response unit/resonance unit</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
</tr>
<tr>
<td>STI</td>
<td>sexually transmitted infection</td>
</tr>
<tr>
<td>TB</td>
<td>terrific broth</td>
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</table>
TBS  tris buffered saline
TBST  tris buffered saline + Tween 20
Tdf  TonB-dependent function
TdT  TonB-dependent transporter
TEV  tobacco etch virus
TGF-β  transforming growth factor beta
Th  T helper
TLR4  Toll-like receptor 4
TMB  3,3',5,5'-Tetramethylbenzidine
TNF-α  tumor necrosis factor alpha
TPEN  N,N,N',N'-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine
U  enzyme unit
UCSF  University of California at San Francisco
VcSLP  *Vibrio cholerae* surface lipoprotein
WCL  whole-cell lysate
WHO  World Health Organization
WT  wild type
x g  times gravity
Zn/Zn²⁺  zinc
ZnSO₄  zinc sulfate
zur  zinc uptake regulator
# LIST OF AMINO ACID SHORTHAND CODES

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<td>Aspartic Acid</td>
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<tr>
<td>Tyrosine</td>
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<tr>
<td>Valine</td>
<td>Val, V</td>
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PREFACE

Before you lies the dissertation entitled “Functional and Immune Characterization of the Gonococcal Zinc Importer TdfJ”, the majority of which encapsulates my research efforts in the laboratory of Dr. Cynthia Nau Cornelissen during my PhD training at Georgia State University. I conducted the research described herein between March 2019 and June 2022, after having already completed a master’s thesis on the same topic between August 2016 and February 2019. This document was written in July 2022.

I chose to enter a research-based master’s degree program after having spent many years with the intention of going to medical school to become a physician. After realizing that medical practice was not the correct fit for me, and subsequently gathering considerable experience in laboratory settings through internships and undergraduate programs, graduate study seemed to be the obvious choice. Roughly halfway through my master’s, I made the decision that I should pursue a PhD afterwards, with the expectation that I would parlay it into a senior research position in industry or academia. I was fortunate to have landed in a great laboratory with a mentor who was welcoming and made me want to stay, so I continued with the same group (although we moved to a new school over 500 miles away in the process!) for my PhD. As I sit here now, writing about my experience and thinking about what comes next, I’m met with a bittersweet mixture of emotions. I’m sad to be leaving this behind after it has so profoundly shaped my last 6 years, but also excited for what’s next, and happy to think I’ve made a difference. I’m sure the Germans have a very long word for this.

I hope you enjoy your reading.

SM
CHAPTER 1: INTRODUCTION

I. Family Neisseriaceae

Upon its first proposal in 1933, the family Neisseriaceae contained the genera Neisseria, Acinetobacter, Moraxella, and Kingella. Cumulatively, these genera consisted of Gram-negative, aerobic or facultatively anaerobic, oxidase- and catalase-positive bacteria which did not form spores. No colony morphology was specified, and members were either rod-shaped or coccoidal (1). Since its inception, the family has been revised several times, with Moraxella and Acinetobacter having been removed and placed into the family Moraxellaceae based on analysis of the genera’s ribosomal nucleic acids (2, 3). Similarly, new additions Alysiella (4), Eikenella, and Simonsiella (5) today complete the family, which constitutes a considerable branch of the β-proteobacteria.

Within the family, Kingella and Eikenella are predominated by human commensals found in the bowel and oral cavity, which rarely cause opportunistic infections of the joints, bones, and tendons. Neisseria, named for German bacteriologist Albert Neisser after its discovery in 1879, are non-motile diplococci with flattened sides, an optimum growth temperature between 35-37°C, and a requirement for sufficient CO₂ presence (1, 6). Several members of the Neisseria are human commensals, and two members, N. meningitidis and N. gonorrhoeae, are human-specific pathogens which, despite their considerable similarity at the genome level, cause markedly different diseases. Human-associated Neisseria can be identified via acid production from glucose or maltose, nitrite reduction, and polysaccharide production from sucrose (7, 8). A notable exception to nitrite reduction by the Neisseria is exemplified by N. mucosa, which is unique in its ability to reduce nitrate to nitrite.
II. Pathogenic *Neisseria*

While both *N. meningitidis* and *N. gonorrhoeae* are human specific pathogens, *N. meningitidis* can be carried asymptotically, frequently colonizing the nasopharyngeal epithelia of healthy individuals (9). Occasionally, the meningococcus will cause severe disease following dissemination to the brain and meninges. By contrast, the gonococcus is always considered pathogenic and not part of the normal flora. It most often colonizes the mucosa of the urogenital system, the rectum, and the oropharynx/nasopharynx, though dissemination is possible. Curiously, there is little to separate the two species at the genome level, despite their considerable difference in disease outcomes; the primary feature to distinguish the two morphologically is the presence of an antiphagocytic polysaccharide-based capsule in *N. meningitidis* (10). Although most other *Neisseria* are commensal and can be carried without causing disease, they occasionally cause opportunistic infections in those with weakened immune systems, which include outcomes such as endocarditis, otitis, septicemia, and meningitis (11).

III. Meningococcal Disease

A. Disease

*N. meningitidis* uses outer-membrane pilin and opacity proteins to facilitate adhesion and invasion of the nasopharyngeal mucosa. These adhesins stimulate the epithelia into engulfing the bacteria into phagocytic vacuoles, which may then traverse to the subepithelial tissue (12). From this point, the meningococcus may gain access to the bloodstream and disseminate, causing systemic disease (13). The resulting bacteremia may lead to colonization of the meninges, pericardium, and synovial joints (14), with seeding in
the meninges typically resulting in meningitis. A typical meningitis patient presents with fever, neck stiffness, photophobia, nausea, and a severe, sudden-onset headache (15, 16), and must be treated immediately to minimize life-threatening disease progression. Up to 75% of meningitis patients show bloodstream colonization by *N. meningitidis*, but sepsis is comparatively rare, occurring in <20% of patients (16). Meningococcal sepsis, usually called meningococcemia, can result in development of hypotension and a purpuric rash, and may progress to renal hemorrhage and organ failure (15). In addition, invasive meningococci can cause pneumonia in up to 15% of patients, though detection is complicated by the high incidence of asymptomatic carriage.

**B. Epidemiology**

Approximately 10% of humans are asymptomatic carriers of *N. meningitidis*, where it resides as a member of the normal flora of the nasopharynx (17, 18). Within that 10%, roughly 40% are colonized transiently, and approximately 25% are colonized for months at a time (19). Carriage rates as high as 90% have been reported during epidemic outbreaks (17), and even 100% in closed environments such as school dormitories and military barracks (20). Carriage is closely associated with age; rates are typically low after birth, then increase dramatically during adolescence and peak in early adulthood (21). Transmission is a result of direct person-to-person contact or direct contact with nasal and/or oral secretions (22), and factors such as tobacco smoke exposure are correlated with increased carriage and risk of disease (23). In addition, those with complement defects are at elevated risk of recurrent infections and more serious disease outcomes (24, 25). Incidence of meningococcal disease is variable, with case burden ranging from as low as 1 to as high as 1000 cases/100,000
people/year, with location as a primary factor (20). Aside from location, seasonal variation of disease has been observed. In the so-called “meningitis belt” of Africa, flanked by Ethiopia to the east and Senegal to the west, epidemics arise at the end of the dry season and reach levels of 1000 cases per 100,000 people (26). Lastly, disease outbreaks are variable between meningococcal serogroups, which are determined by the aforementioned polysaccharide capsule. In Europe, the Americas, Australia, and New Zealand, serogroup B dominates, with serogroup C behind, causing <10 cases per 100,000 each year. By contrast, serogroup A has emerged as the primary cause of outbreaks in Africa, which occur roughly every 5-10 years, and most of Asia (27). In total, serogroups A, B, C, W-135, X, and Y are responsible for over 90% of meningococcal disease (20).

C. Treatment and Prevention

Before the advent of antimicrobial therapy, meningococcal infection carried a case fatality rate of approximately 70% (28). However, in modern times thanks to medical advances, mortality rates have been greatly reduced, hovering at around 9-12% on a per infection basis. For those with meningococcemia, this is as high as 40% (15). Despite the considerable reduction in mortality rates, however, convalescent patients still experience serious, life-altering complications such as loss of hearing, loss of limb(s), and neurological disability in 11-19% of cases (14, 29). The first described antimicrobials to treat meningitis were the sulfonamides, which began use in the 1930s. However, resistance to these drugs quickly led to a switch to penicillin, which remains the preferred therapy to this day (15, 30). Owing to the severe nature of meningococcal infection, containment and prevention strategies are paramount. Caretakers, frontline medical staff, and others in close proximity
to infected patients are at elevated risk for exposure and are frequently prescribed prophylactic drugs to minimize risk. Ideally, these antibiotics will altogether eliminate meningococcal carriage in these persons, and typically consist of ceftriaxone, ciprofloxacin, and rifampin. Despite resistance to sulfonamides arising relatively early, resistance to other drugs is rare in *N. meningitidis*, though there is some evidence that it is emerging (30-33).

As with many infectious diseases, vaccination has been the gold standard for preventing meningococcal infection worldwide. The MPSV4 vaccine, produced by Sanofi Pasteur, has been available since 1981 and targets the polysaccharide capsules of *N. meningitidis* serogroups A, C, Y, and W-135. In adults, MPSV4 is 85% effective at preventing meningococcal disease (34), but the product’s nature as a polysaccharide-only vaccine causes some limitation in its overall effectiveness. The vaccine is not known to stimulate T-cells, and therefore generates no immunological memory and has no impact on carriage, while antibody titers decline in the absence of a booster (35). Recently, these shortcomings have been addressed via the introduction of protein conjugate vaccines, such as MenACWY (Menactra) that can elicit a T-cell response (36, 37). Today, MenACWY vaccination is recommended as part of a child’s routine vaccination (38).

A notable omission from the quadrivalent vaccines described above is *N. meningitidis* serogroup B, which accounts for 32% of infections in the United States and up to 64% in Europe, New Zealand, and Australia (39). Vaccine development for serogroup B was unable to target the polysaccharide capsule due to the structure of its sialic acid. With the exception of serogroup A, meningococcal capsules are composed of sialic acid derivatives, and serogroup B utilizes sialic acids with α2-8 linkages identical to those found...
in many human tissues (40-42). This molecular mimicry threatened to reduce vaccine immunogenicity due to tolerance, and may have induced autoimmune activity against a self-antigen, so the capsule was avoided (43). As an alternative, other outer-membrane structures for serogroup B were targeted, eventually resulting in a four-component vaccine consisting of Neisseria adhesin A (NadA), Neisseria heparin binding antigen (NHBA), and factor H binding protein (fHbp), presented alongside a detoxified outer-membrane vesicle from the New Zealand outbreak strain NZ98/254 (4CMenB/Bexsero, GSK), which was licensed in the United States in 2014 (39). Depending on the antigenic presentation of the target strain, notably the subtype of PorA, 4CMenB is between 80-96% effective (44, 45) at meningococcal killing. Another serogroup B vaccine, Trumenba (Pfizer), is also licensed in the United States as of 2014 (39), and uses two variants of fHbp. These platforms have not been associated with safety concerns other than mild reactions at the injection site (46-48), and currently, the recommendation in the United States is vaccination against serogroup B at age 16.

IV. Gonococcal Disease

A. Disease

Gonorrhea, caused by its namesake, N. gonorrhoeae, takes its name from the Greek words gonos, meaning “seed”, and rhoe, meaning “flow”, owing to early physicians misidentifying the characteristic purulent discharge as semen (49). Primary gonococcal infections occur in the urogenital, rectal, pharyngeal, and conjunctival tissues (50). In men, infection typically presents as urethritis and dysuria, characterized by a neutrophil-rich urethral discharge. Uncommonly, this infection can be asymptomatic, and may progress to
epididymitis or prostatitis if left untreated (51). For women, the usual presentation is cervicitis, but unlike in men, infection can be asymptomatic in over 50% of cases. When untreated, ascending infections present a major threat, potentially causing salpingitis, pelvic inflammatory disease, and damage to the fallopian tubes which may lead to infertility or ectopic pregnancies (52). Further, those who carry asymptomatic infections are at risk for secondary complications from dissemination, including septic arthritis, endocarditis, and meningitis (53-56). While genital infections are the most common, gonococcal disease may also arise in the mouth, throat, rectum, and eyes, which are often difficult to diagnose and treat (57, 58).

B. Epidemiology

Gonorrhea is the second-most-commonly reported infectious disease in the United States, with over 600,000 cases reported in 2019 and nearly 100 million estimated cases worldwide per year (59). Direct medical costs associated with treating gonococcal infections in the United States have reached as high as $271 million (60). N. gonorrhoeae typically spreads through direct sexual contact, and like N. meningitidis, age is a strong determinant. Teenagers and young adults between ages 15-29 represent the highest incidence of gonococcal infection, but other factors such as race, socioeconomic status, sexual orientation, and number of sexual partners are notable as well (60, 61). For example, Native Americans, Latinos, and those of African descent represent a disproportionately large percentage of overall infections, and infections among men who have sex with men increase yearly. There is a strong association between gonorrhea and HIV infection, as gonococcal infection has been shown to increase HIV viral replication and gene expression.
Similarly, co-infection with *Chlamydia trachomatis* is common among those carrying gonococcal infections (65). As mentioned above, infected women are frequently asymptomatic, which is believed to make them consistent reservoirs of *N. gonorrhoeae* within the population (66, 67). As a consequence, more cases of gonorrhea are reported in men than in women.

C. Treatment and Prevention

Unlike *N. meningitidis*, the gonococcus has a remarkable propensity to acquire and maintain resistance to antimicrobial drugs, and there is considerable evidence to suggest that our current treatment options may soon be rendered ineffective (68-74). Initially, like the meningococcus, infection by *N. gonorrhoeae* was treated with sulfonamides, but less than a decade later high levels of resistance had emerged, necessitating a switch to penicillin. In the roughly 80 years since, resistance to every class of antimicrobial drug utilized for treating gonorrhea has emerged (75), complicating the outlook for future treatment options. Because the WHO and CDC have recommended that treatment have a high cure rate with a single dose, and because co-infection with Chlamydia is common, the CDC-recommended therapy for gonococcal infections was, until recently, a one-time dual therapy of 250 mg intramuscular ceftriaxone co-administered with 1 g oral azithromycin (74). However, due to increased resistance and its long subtherapeutic tail, azithromycin was dropped from this recommendation in December 2020 (76). Alternative combinations have been trialed with a few promising results, including 2 g azithromycin combined with either 240 mg gentamycin or 320 mg gemifloxacin (77), which has been recommended for infections with low β-lactam sensitivity. While vaccination efforts to control meningococcal
disease have been largely successful, the same is not true for gonorrhea. To date, no effective vaccine to prevent gonococcal infections has been identified, and natural infection does not elicit a protective immune response. For now, prevention and control of gonorrhea is limited to lifestyle choices such as safe sex practices, and effective screening and contact tracing to limit outbreaks.

V. Virulence Factors of Neisseria gonorrhoeae

Owing to centuries of co-evolution alongside the human host, N. gonorrhoeae possesses a considerable arsenal of virulence factors that contribute to adhesion, invasion, dissemination, nutrient acquisition, immune suppression, and immune evasion. Many of these key factors are surface structures which are critical to interactions with host cells at the initial stages of infection, a summary of which is shown in Figure 1. Contributing to their importance in infection is the fact that many of these genes are subject to either phase or antigenic variation at high frequency, which are major factors in immune evasion.

A. Type IV Pilus

The type IV pilus is a common feature of Gram-negative bacteria, and the gonococcus in no exception. The type IV pilus is comprised of a filamentous polymer that protrudes from the cell surface. This structure is 6 nm in diameter and may extend up to several microns from the bacterial cell (78). Pilin synthesis is divided into 4 steps: assembly, functional maturation, counter-retraction, and emergence on the cell surface, which are completed by 23 separate genes (79, 80). In total, the pilus is composed of 15 proteins, with varying roles in biogenesis, assembly, and disassembly of the overall structure (80). The major structural subunit of the pilus is PilE, a ~20 kDa protein which assembles into a
Figure 1. Overview of surface-associated virulence factors in *Neisseria gonorrhoeae*

A cartoon image of gonococcal cells demonstrating surface-exposed virulence factors, accompanied by a brief description of their function. Adapted from (81).
helix to generate the overall filament. Further, the minor pilin proteins PilC, PilV, and PilX can be incorporated into the mature pilus to modulate its function (82, 83).

The pilus plays multiple roles in gonococcal pathogenesis. Primarily, it acts as a major adhesin for attachment to the mucosal epithelia (78, 84), and is also active in microcolony formation (82). In addition, *N. gonorrhoeae* is naturally competent thanks to the ability of the pilus to facilitate uptake of naked DNA from outside the cell. This lateral gene transfer is thought to be a major contributor to development of antimicrobial resistance, and to the genetic diversity of gonococcal strains (85, 86). Finally, rapid assembly and disassembly of the pilus through the coordinated actions of PilT and PilC facilitates a quasi-motility – the so-called “twitching motility” (87).

Gonococcal pili are subject to antigenic variation at high frequency. In this process, a non-reciprocal, RecA-dependent exchange occurs between promoterless “silent” pilS genes and the expressed pilE gene, resulting in antigenically distinct gene chimeras. This conversion is thought to occur with a frequency of once per 100 cells (88, 89). Beyond this, pilE is also subject to phase variation via a poly-C tract within its coding region, which may slip out of frame during DNA replication resulting in decreased pilus production.

**B. Opacity Proteins**

The opacity proteins, or Opas, are a closely related family of transmembrane proteins in the outer membrane of *N. gonorrhoeae*, named originally for their distinct effects on the opacity of gonococcal colonies when viewed on solid media (90). The Opas consist of an eight-stranded β-barrel which generates four surface-exposed loops (78). A typical gonococcal chromosome contains 11 *opa* loci whose gene products enable invasion
of epithelial cells and leukocytes (91-94). The Opas are thought to play an essential role in gonococcal infections, which is exemplified by their expression in cultures recovered from infected male patients (95) and in experimental urethral infections (96).

Opas are largely categorized by their tropism for receptors within the human host. The Opas recognize two distinct sets of host receptors. First are the carcinoembryonic antigen-related cell adhesin molecules (CEACAMs) (97, 98), and second are heparin sulfate proteoglycans (99, 100). Indeed, the specific complement of Opas expressed by a gonococcal strain affects disease outcome. For example, binding of vaginal CEACAM5 is associated with long-term colonization in the lower genital tract, while binding CEACAM1 enhances penetration of the epithelial tissues and may contribute to dissemination (101). Paradoxically, Opa binding of CEACAM3 on neutrophils is associated with enhanced phagocytic killing of gonococci (102), although recent studies have suggested an evolutionary pressure for *N. gonorrhoeae* to minimize this interaction (103). Beyond their role as invasins, the Opas are known to modulate the host immune response in a multitude of ways. Opa binding to CEACAM1 has been shown to arrest activation and proliferation of CD4+ T-cells (104), and can cause death of B-cells (105). Opas have also been implicated in the ability of the gonococcus to skew the host immune response towards a pathogen-favorable Th17-biased outcome and away from Th1 and Th2, which is primarily mediated through upregulation of TGF-β and IL-10 (106, 107).

Like the type IV pilus, the Opas demonstrate high frequency antigenic and phase variation. Every *opa* gene contains a pentameric repeat [CTCTT]_n within a 5’ section of the gene that encodes a leader peptide. Stochastic slipped-strand mispairing within this repeat
section facilitates phase-variable expression of the opas, allowing a population of gonococci to express multiple opas, a single opa, or no opa at any given time (108-110).

C. Lipooligosaccharide

Like other Gram-negative bacteria, *N. gonorrhoeae* produces a major glycolipid on its cell envelope. In most species, this glycolipid is lipopolysaccharide (LPS), a highly immunogenic structure consisting of a membrane anchor domain called Lipid A, a short oligosaccharide core, and a polysaccharide consisting of repeating O-antigen, which extends away from the cell surface at variable length. However, the pathogenic *Neisseria* lack the O-antigen domain, resulting in a truncated glycolipid called lipooligosaccharide (LOS) (111). In addition to its usual role as an endotoxin, LOS plays key roles in gonococcal virulence. For example, it can bind the asialoglycoprotein receptor in human sperm, which increases invasion of the urethral epithelia (112, 113), and can mimic human glycosphingolipids (114). Certain LOS variants are able to function as acceptors for deposition of sialic acid via activity of gonococcal sialyltransferases (115-117). During gonococcal infection, host-derived cytidine-5’-monophospho-N-acetyleneuraminic acid (CMP-NANA) functions as a sialic acid donor (118, 119), which contributes to serum resistance (120). Beyond sialylation, LOS can coat itself with phosphoethanolamine (PEA), which provides the gonococcus with resistance to bacterially derived antimicrobial peptides, induces production of proinflammatory cytokines, and is associated with resistance to complement-mediated killing (121-124). Indeed, gonococcal strains which lack the PEA phenotype show reduced colonization in experimental mouse infection (123). LOS has also been shown to manipulate host dendritic cells, skewing the immune response (125).
Like many other gonococcal surface structures, LOS is subject to variation. This is mediated by phase variability in glycosyltransferase genes responsible for extension of the LOS carbohydrate polymer (126). The genes lgtA, lgtC, and lgtD contain poly-G tracts within their coding regions, which allows for strand slipping and variation in the terminal sugars of the polymer, contributing to antigenic diversity (127). Researchers have suggested that variability of LOS contributes to gonococcal fluctuation between invasive and serum-resistant states, as LOS expression interferes with invasion of certain tissue types (128).

D. Porin

The most abundant outer-membrane protein in N. gonorrhoeae is porin, which constitutes roughly 60% of the membrane’s total protein makeup (129). The porins function as outer-membrane channels and are essential for gonococcal survival (130). Structurally, porins are trimeric complexes, with each monomer forming a β-barrel (131, 132). Interestingly, porin production differs between N. meningitidis and N. gonorrhoeae. The meningococcus produces two classes of porin: PorA, the larger class at roughly 45 kDa, and PorB, the smaller class at roughly 35 kDa (133, 134). In the gonococcus, however, porA is a pseudogene incapable of being expressed, resulting in only production of PorB (135, 136). This PorB is further classified into two subclasses: PorB1A, and PorB1B (135). These subclasses are associated with differing disease outcomes, with PorB1A being strongly associated with disseminated phenotypes, and PorB1B primarily being found in localized infections (137).

In addition to their importance to cell viability, the porins play key roles in pathogenesis. Porins are known to translocate into host cell membranes, which may disrupt
host cell signaling and prevent phagosome maturation and degranulation, which is thought to promote intracellular survival by phagocytosed gonococci (138-140). PorB1A has been shown to interact with host Factor H and C4b binding protein, which leads to serum stability and resistance to the classical and alternative complement pathways (141-143).

E. IgA1 Protease

IgA is the predominant subclass of immunoglobulin in the human mucosa, the site of most Neisseria infections. To combat this, Neisseria species produce a proteolytic enzyme to cleave IgA and neutralize its impact (144, 145). The IgA1 protease is secreted in its immature form, after which it matures via a series of endoproteolytic cleavage steps (146). Once mature, IgA1 protease specifically targets the proline-rich hinge region of the IgA1 heavy chain, although the exact cleavage site is determined by which class of IgA1 protease is expressed: the type 1 enzyme cleaves a proline/serine bond at residue 237, while class 2 cleaves targets a proline/threonine bond at residue 235 (145, 147, 148). IgA1 protease is incapable of cleaving IgA2.

Interestingly, IgA1 protease also cleaves lysosome-associated membrane protein 1 (LAMP1), a glycoprotein common in lysosomes and late endosomes (147). LAMP1 cleavage causes accelerated lysosomal degradation, which is thought to aid intracellular survival of Neisseria (149). While IgA1 protease is not required for gonococcal colonization, there is some suggestion that it plays a key role in transcytosis, as a mutant N. gonorrhoeae not expressing the iga gene is defective for this function (149, 150).
VI. Host Metal Restriction and Homeostasis

Like all bacteria, the Neisseria require sufficient access to trace metals such as iron and zinc as part of their normal metabolism. As such, the ability to acquire these metals has been deemed an important contributor to pathogenesis for infectious microbes (151-153). In response, the human host has evolved mechanisms to restrict iron and zinc availability, effectively starving pathogens of key nutrients and handicapping their infective potential. This strategy, called “nutritional immunity” is broadly effective against many pathogens (154-156).

A. Iron

Iron has been noted as a key catalytic element in enzymatic proteins, and as a strong electron acceptor. It is a critical component of biological processes such as respiration and nucleoside biosynthesis, and is frequently found in structural complexes such as iron-sulfur clusters (157). Elemental iron exists in two redox states which are readily interchangeable: ferrous (Fe$^{2+}$) and ferric (Fe$^{3+}$). Despite its nature as an essential nutrient, iron chemistry can have harmful biological outcomes through the Fenton reaction. In its ferrous form, iron can cause creation of damaging hydroxyl radicals in the presence of oxygen (158, 159).

Due to its propensity for toxicity and damaging Fenton chemistry, iron regulation in the aerobic environment of the mammalian body is paramount (160). The primary effectors for iron sequestration are iron-scavenging extracellular transport proteins, and intracellular ferritin, which cumulatively make free iron extremely scarce ($\sim 10^{-18}$ M in plasma) (161). A byproduct of this tight regulation is restricted access to iron by invading pathogens (155, 162, 163).
1. **Transferrin**

One of the most preeminent iron transporters in humans is transferrin, a glycoprotein of roughly 80 kDa found in abundance in the serum, through which it transports iron to various tissues (164). Transferrin is a bi-lobed protein, one N-terminal and one C-terminal, and each lobe contains a high-affinity iron binding pocket, capable of coordinating a single Fe³⁺ with a dissociation constant of roughly $10^{-22}\text{M}$ (165, 166). Transferrin concentration in human serum ranges from ~25 to 50 μM, and of the total available iron binding sites in the circulating transferrins, roughly 30% contain an iron atom at any given time, with the N-lobe pocket being slightly preferred (164, 167, 168). All human cells with a metabolic requirement for iron produce the transferrin receptor, which preferentially binds ferrated transferrin (diferric transferrin binds more tightly, but both monoferric forms make stable complexes with the receptor) (169). After binding, the transferrin-receptor complex is internalized into an endosome, which liberates iron via acidification, leaving an apo-transferrin-receptor complex to be recycled to the cell surface. Once on the surface, the apo-transferrin is displaced by a new ferrated transferrin molecule, which binds the receptor with higher affinity than the apo form at neutral pH (170, 171).

2. **Lactoferrin**

Lactoferrin, a member of the transferrin family, is less abundant in serum, and instead is enriched in secretions such as saliva, semen, vaginal fluids, urine, and bile. (172, 173). In milk, it is the second most abundant protein behind casein, and can also be found in amniotic fluids, blood plasma, and certain leukocytes (174-177). Like transferrin, lactoferrin is a bi-lobed glycoprotein roughly 80 kDa in size, and it binds iron with high affinity (178,
In addition to iron, various reports have demonstrated lactoferrin binding to other metals such as copper, manganese, and zinc (180). Because lactoferrin is found in such a wide array of anatomical sites, and because it retains its iron binding capacity over a large range of pH, it is particularly well suited to not only host iron homeostasis, but bacterial growth suppression as well. This is accomplished via two mechanisms. The first, unsurprisingly, is via iron sequestration, but in addition, the N-terminus of lactoferrin is cleavable via autoproteolysis, resulting in the production of small antimicrobial compounds called lactoferricins (181-183). These molecules interact with bacterial surface receptors to cause membrane destabilization through charge disruption, ultimately causing cell lysis.

3. Heme and Hemoproteins

A small porphyrin ring that coordinates ferrous iron, heme is responsible for storing approximately 70% of human iron (184). Heme plays a key role in oxygen chemistry, including the oxidative stress response, oxygen storage and transport, and electron transport (185). Paradoxically, this essential compound’s high catalytic potential and lipophilic tendencies quickly lead to toxicity when heme is abundant, and as a counter measure, approximately 95% of human heme is sequestered in hemoproteins, of which 67% is hemoglobin. Hemoglobin primarily resides in erythrocytes and exists as a tetramer of two α and two β subunits, and in total binds four heme molecules (186). While hemoglobin is the predominant reservoir for heme, the hemoglobin molecule is subject to hemolysis, resulting in circulating αβ dimers, which are themselves eventually sequestered by haptoglobin to minimize toxicity (187-189). Damage to hemoglobin and other hemoproteins
results in liberation of heme molecules which must quickly be neutralized, and this function is usually performed by albumin and hemopexin.

4. Ferritin

While the previously mentioned iron scavengers primarily control extracellular iron, excess intracellular iron is sequestered by ferritin. The ferritins are heteropolymeric compounds composed of 24 subunits which fall into two categories: heavy (H) and light (L). Both H and L chains are required to produce a fully functional ferritin, and the final structure is a 450 kDa spherical shell capable of accommodating up to 4500 iron atoms (190-192). Ferritin upregulation is indirectly associated with inflammation and infection, owing to the fact that serum iron decreases in this situation, increasing cellular iron (193). Liberation of iron from ferritin is accomplished primarily through proteasomal degradation when intracellular iron pools become scarce, effectively making ferritin a large iron repository for the cell (194). The importance of the ferritins in mammalian survival is exemplified by ferritin knockout mice, which are nonviable (195).

B. Zinc

Similarly, zinc plays essential roles in cellular processes including signaling, catalysis, and as a structural component of metalloproteins (152, 196). Zinc is stable in the 2+ redox state, and is known to act as a preferred Lewis acid when activation of poor nucleophiles is necessary (197). It is estimated that approximately 6% of bacterial proteins contain zinc, of which 80% are enzymatic in nature. In eukaryotic cells, zinc is distributed fairly evenly, with roughly 50% in the cytoplasm, 40% in the nucleus, and the remaining 10% in membranes
and/or cell walls (198). For humans, an estimated 3000 metalloproteins and enzymes utilize zinc as a structural or catalytic element (199).

Like iron, levels of zinc are tightly regulated to control unwanted zinc-mediated reactivity. For example, excess zinc can inhibit clathrin-mediated insulin endocytosis, causing dysregulation of insulin clearance (200). Total intracellular zinc typically ranges in the hundreds of micromolar, but the vast majority of this is sequestered in proteins or dedicated zinc stores, leaving free zinc at picomolar concentrations (201).

1. **S100 Proteins**

The S100 family of proteins, named for their solubility in saturated ammonium sulfate, are EF-hand-containing proteins primarily responsible for calcium signaling and homeostasis in mammals (202, 203). Beyond their role in calcium regulation, the S100s participate in other intracellular processes such as cell cycle regulation, transcription, motility, and cell growth and differentiation. Some also serve as important markers of disease such as cardiomyopathy and neurodegeneration (204). Over 20 members of the S100 family have been identified, each of which is made up of the same basic structural unit: a helix-Ca\(^{2+}\) binding loop-helix motif, which packs into pairs to create a stable four-helix bundle (205). Beyond this, the S100s are obligate dimers, with each four-helix monomer integrating into a highly-stable antiparallel dimer with eight helices (206). All S100 proteins can form this eight-helix bundle as a homodimer, which is the preferred orientation; however, heterodimerization is possible, and indeed the S100A8/S100A9 heterodimer is unique in its preference to form the heterodimer (207). In some S100
species, higher-order oligomerization is possible, primarily mediated by formation of disulfide bridges.

The S100s also play important roles in immune modulation, especially with regard to regulating inflammation (208). S100s have been shown to demonstrate both cytokine- and chemokine-like activity, primarily through interactions with pattern recognition receptors such as the receptor for advanced glycation end products (RAGE) and Toll-like receptor 4 (TLR4) (208-210). These interactions drive a variety of responses, including macrophage, neutrophil, and monocyte recruitment (210-212), as well as activation of proinflammatory cytokines via NF-κB and p38 MAPK pathways (213, 214). Conversely, the S100s also exhibit many anti-inflammatory properties, including scavenging excess reactive oxygen species (215) and promoting apoptosis of tumor cells and damaged leukocytes and endothelial cells (216, 217). Perhaps owing to their immunomodulatory capabilities and association with inflammation, several S100s are associated with inflammatory diseases. For example, S100A7 is found in abundance in inflamed skin and is associated with inflammatory skin conditions such as atopic dermatitis and psoriasis (218, 219), from which it takes its common name “psoriasin.” S100A12 and calprotectin (the S100A8/S100A9 heterodimer) are associated with conditions such as inflammatory bowel disease, cystic fibrosis, and rheumatoid arthritis (220-222).

Finally, a unique characteristic of the S100s which distinguishes them from other EF-hand proteins is the formation of two transition metal binding sites at the dimer interface (223-225). This metal sequestration ability grants several S100s an auxiliary role as nutritional immunity proteins, which curtail bacterial growth through nutrient restriction
The homodimers S100A7 and S100A12 contain symmetrically disposed binding sites. In S100A7, the sites are comprised of residues H17 and D24 from one monomer and H86 and H90 from the other, which facilitate Zn\(^{2+}\) ligation at high affinity (224). In S100A12, both Zn\(^{2+}\) and Cu\(^{2+}\) can be coordinated by binding pockets comprised of H15 and D25 of one monomer, and H85 and H89 from the other (225, 227). The heterodimer calprotectin possesses two unique binding sites. Site II exhibits similar geometry and Zn-binding activity to the S100A7 sites, and contains H83 and H87 from S100A8, and H20 and D30 from S100A9 (228, 229). Site I is capable of binding both Zn\(^{2+}\) and Mn\(^{2+}\) with high affinity \(K_d (\text{Zn}^{2+}) \sim 10^{-9} \text{M, } K_d (\text{Mn}^{2+}) \sim 10^{-7}-10^{-8} \text{M}\), but coordination differs depending on which ion is present. For zinc, residues H17 and H27 from S100A8 and H91 and H95 from S100A9 are sufficient. However, Mn\(^{2+}\) ligation requires octahedral geometry, which is accomplished via the contribution of the previous four histidines in addition to two extra His residues on the C-terminal tail of S100A9, creating a unique His\(_6\) site not seen in other Mn-binding proteins (223, 230). Cumulatively, S100A7, S100A12, and calprotectin have demonstrated antimicrobial activity against pathogens such as *Staphylococcus aureus*, *Helicobacter pylori*, *Candida albicans*, *Acinetobacter baumannii*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella enterica* serovar Typhimurium via their metal restriction functions (219, 231-243).

2. Metallothioneins

Due to the relatively low Ca\(^{2+}\) concentration in the intracellular environment, the S100s primarily function extracellularly. As such, zinc homeostasis within mammalian cells requires another mediator. The metallothioneins, which are small, cysteine-rich proteins,
fulfill this role in much the same manner as the ferritins for intracellular iron, though on a much smaller scale; a single metallothionein can coordinate up to seven zinc ions (244, 245). It is estimated that between 5 and 15% of cytosolic zinc is bound by the metallothioneins, while other zinc is frequently shuttled in and out of endosomes (196, 246).

**VII. Metal Acquisition by the Pathogenic Neisseria**

As obligate human pathogens, *N. meningitidis* and *N. gonorrhoeae* have evolved to be superlatively adapted to survival within this host niche, including with regard to acquisition of critical trace metals. The primary mechanism by which this is accomplished is via production of high-affinity TonB-dependent outer-membrane transporters (TdTs), which are accompanied by the Ton motor complex and dedicated ABC transporters which shuttle the metal cargo from the outer membrane across the periplasm and into the cytoplasm. An overview of the gonococcal TdTs, the Ton motor, and the cognate ABC transporters is shown in Figure 2.

**A. Two-component TonB-dependent Transporters**

The gonococcus produces a total of eight TdTs, all of which share the same overall structure. The standard TdT is comprised of a 22-stranded amphipathic β-barrel which spans outer membrane to produce a traversable channel, 11 flexible extracellular loops which constitute the regions between antiparallel strands of the barrel, and a folded plug domain at the N-terminus, which occludes the pore of the barrel (247, 248). Three gonococcal TdT systems utilize a second component to complement the transporter: a lipid-modified accessory protein tethered to the outer membrane, which increases the efficiency
Figure 2. TonB-dependent transporters, surface-tethered lipoproteins, and ABC transport systems produced by *Neisseria gonorrhoeae*

Cartoon schematics of the eight known TdTs produced by the gonococcus are represented by barrel shapes in the outer membrane, and their respective ligands identified to date, shown by green hexagons. A zinc ion bound to TdfJ is highlighted in yellow. Also illustrated are the lipoprotein components associated with TbpA, LbpA, and HpuB, shown here anchored to the outer membrane. TonB, ExbB, and ExbD, which utilize the proton motive force to energize the transport of cargo through the outer membrane via the TdTs are shown in the left-hand side of the inner membrane. Black squiggles on the outer leaflet represent lipooligosaccharide. Lastly, the cognate ABC transport systems associated with the TdTs are shown in the inner membrane and the periplasmic space. Ligands: Tf = Transferrin; Lf = Lactoferrin; Hb = Hemoglobin; Xs = Xenosiderophore; Cp = Calprotectin. Adapted from (249).
of transporter function. The first such system is the transferrin-iron uptake system, which binds the C-lobe of human transferrin and strips its iron (250, 251). The first component is the transmembrane barrel, transferrin binding protein A (TbpA), which binds both apo- and Fe-transferrin and facilitates iron passage across the outer membrane. This iron piracy depends on at least three motifs within TbpA. The first, a protruding α-helix finger in extracellular loop three, acts at the TbpA-transferrin interface. Upon transferrin docking, this helix inserts into the Fe-binding cleft of the transferrin C-lobe, where it is thought to disrupt iron ligation (252, 253). Mutagenesis of this region resulted in disruption of both binding and iron acquisition from transferrin (254). The second motif, which is present in all TdTs, is a short N-terminal amino acid sequence known as the TonB-box, which interacts with the TonB protein from the Ton motor complex to energize the transporter (255). Mutagenesis of this region has no effect on transferrin binding, but results in abrogation of nutrient import. Third is another amino acid motif in the plug domain, which consists of the residues EIEYE. This negatively-charged motif is hypothesized to transiently chelate the pirated iron atom after its extraction from transferrin, and is necessary for iron utilization (256, 257).

The second component, TbpB, is a bi-lobed lipoprotein with specificity for Fe-transferrin, which binds exclusively to the TbpB N-lobe. While not strictly necessary for transferrin utilization, TbpB greatly increases the efficiency of iron import by TbpA (252, 258-261). The *tbpAB* system is encoded in the genome of all gonococcal and meningococcal strains (262), and is arranged into an iron-regulated bicistron, with *tbpB* upstream of *tbpA* (263). TbpA is very well conserved, with gonococcal isolates showing >95% sequence
identity (264). Conversely, TbpB is fairly antigenically variable (265), sparking the hypothesis that it may partially function as an immune “decoy” to protect TbpA. The importance of iron piracy is underscored by an experimental urethral infection study in human males, wherein a gonococcal strain incapable of utilizing transferrin was unable to establish infection (266).

The lactoferrin-iron acquisition system, comprised of the transporter lactoferrin binding protein A (LbpA) and the lipoprotein LbpB, pirates iron from human lactoferrin, and bears considerable similarity to the transferrin system (267, 268). Like the \( \text{tbp} \) genes, \( \text{lbpA} \) and \( \text{lbpB} \) are found in a bicistronic operon with the \( B \) gene upstream. The precise atomic structure of LbpA is not yet known, but its high level of similarity to TbpA has generated predicted structures with high confidence (269). These models suggest the presence of the same critical motifs – loop 3 \( \alpha \)-helix, TonB-box, and EIEYE – as described for TbpA, which are hypothesized to function in a similar manner. Recently, the crystal structure of LbpB in complex with lactoferrin revealed that, like TbpB and transferrin, LbpB preferentially recognizes the ferrated form of the ligand, with the N-lobe of LbpB binding the C-lobe of lactoferrin (270), suggesting a similar role for increasing efficiency of iron uptake by LbpA. Beyond its role in iron acquisition, LbpB also aids in protecting \textit{Neisseria} from the lactoferrin-derived antimicrobial peptide lactoferricin (271). Unlike the transferrin system, however, only \( \sim 50\% \) of gonococcal isolates express the lactoferrin system, and LbpB is frequently phase off through slipped-strand mispairing (272), which has generated questions about the redundancy of the two systems. Critically, an engineered gonococcal strain expressing the lactoferrin system in the absence of the transferrin system (no such organism is known to exist in nature) recovered the lost infection phenotype seen in the \( \text{tbp} \)
mutant, and a strain expressing both systems is at a competitive advantage over those that only express the transferrin system (272).

The third two-component system is the hemoglobin/haptoglobin (Hb/Hp) uptake system, which is found in two forms in the pathogenic Neisseria: HmbR and HpuAB. N. meningitidis is genetically capable of expressing both systems, but to date, all N. gonorrhoeae strains are restricted to production of HpuAB, as hmbR is present only as a pseudogene (273). The hpuAB locus encodes two proteins, the lipoprotein HpuA, and the TonB-dependent transporter HpuB (274, 275), which together facilitate iron acquisition from human hemoglobin and hemoglobin-haptoglobin complexes (274). Unlike the transferrin and lactoferrin systems, production of both components is strictly required for ligand binding and utilization (276). Considerably less is known about the specific mechanisms of iron piracy by HpuAB, and no high-resolution structures of the Neisseria proteins are available. However, modeling of the gonococcal HpuA based on the known crystal structure of HpuA from Kingella denitrificans suggests that gonococcal HpuA is roughly half the size of TbpB and LbpB, and structurally resembles the C-lobe of those proteins (277). The HpuAB system is subject to high-frequency phase variation by virtue of a poly-G tract in hpuA (278), and the utility of this system during infection appears to vary according to the sex of the host. Gonococcal isolates recovered from males are phase-off for the Hpu system. Similarly, most women with localized infections are culture-positive for phase-off isolates, with a notable exception: phase-on isolates can be isolated from women infected during the first two weeks of their menstrual cycle (279). Taken together, these data suggest that HpuAB expression is not necessary for gonococcal colonization, but when
hemoglobin is plentiful, as is the case during menses, the Hb/Hp system is selected, potentially due to high ligand availability.

**B. Single-component TonB-dependent Transporters**

The five remaining TdTs are single-component transporters which lack the accessory lipoprotein. The first, FetA (previously named FrpB) is unique in that its primary nutritional target is not a human protein. Instead, FetA enables the *Neisseria* to utilize siderophores, which are small, non-ribosomal iron chelating compounds commonly produced by other bacterial species (280). The *Neisseria* themselves do not produce siderophores and have evolved this mechanism to hijack the iron scavenging activity of other species, allowing them to utilize siderophores such as ferric enterobactin, ferric salmochelin, and dihydroxybenzoic acid (281, 282). Recently, the solved crystal structure of *Neisseria* FetA revealed a few distinguishing features. First, like the *E. coli* enterobactin receptor, FetA appears to form a trimer, with each monomer existing at about 76 kDa. Second, FetA appears to interact with free iron atoms in addition to iron-loaded siderophores, suggesting some capacity for non-siderophore-associated iron uptake (283). As described for the hemoglobin and lactoferrin receptors, *Neisseria* FetA is subject to phase variation via slipped-strand mispairing. However, unlike the previous examples, the slip is not in the gene’s coding region. Instead, a poly-C tract is present between the promoter’s -10 and -35 region, where extension or contraction via cytosine addition or subtraction changes the strength of the promoter, thereby affecting overall gene expression (284).

The last four TdTs share their nomenclature, and are collectively called TonB-dependent function (Tdf) F, G, H, and J (285). To date, TdfH and TdfJ are by far the better
characterized of the four, while comparatively little is known about TdfF and TdfG. TdfH (called calprotectin binding protein A [CbpA] in *N. meningitidis*) is found in both pathogenic *Neisseria* species but is rare in the commensals. Perhaps unsurprisingly, given its nomenclature in the meningococcus, this 100 kDa protein binds human calprotectin, from which it extracts zinc (286, 287). Recent biophysical studies on the TdfH/calprotectin interaction demonstrated that this binding interaction occurs at nanomolar affinity, and that Site I (the non-canonical Zn$^{2+}$/Mn$^{2+}$ binding pocket) of calprotectin is the target of zinc piracy by TdfH (288), suggesting a potential role for manganese acquisition a well. While this hypothesis has not been directly tested, the cryo-EM structure of TdfH complexed with calprotectin demonstrated that this interaction is possible in the presence of both manganese and copper, in addition to zinc (289). Furthermore, TdfH expression by gonococci mediates survival in neutrophil extracellular traps, which are highly enriched for calprotectin due to its abundance within the neutrophil cytosol (287, 290). TdfH is not known to exhibit any phase or high-frequency antigenic variation.

TdfJ (zinc uptake D [ZnuD] in *N. meningitidis*), unlike TdfH, is found in all *Neisseria* species, both pathogenic and commensal. Early characterization of ZnuD demonstrated its contribution to meningococcal zinc uptake (291), which was later recapitulated for TdfJ in the gonococcus (287). Structural insights into ZnuD revealed two interesting features. The first is an α-helix finger in extracellular loop three, like the one described for TbpA, which is flanked by two His- and Asp-rich clusters of amino acids that are thought to contribute to zinc sensing. The second is a unique, substrate-dependent remodeling by this loop three region. In the absence of zinc, the loop three α-helix retains its helical shape and extends
outwardly from the protein. However, when zinc (or the zinc analogue cadmium) binds a peripheral zinc pocket in this region, the helix rearranges into a pair of flexible β-strands. This rearrangement is thought to facilitate access to a high-affinity zinc site buried within the protein’s β-barrel, which participates in zinc shuttling to the periplasm (292). Curiously, ZnuD/TdfJ shares structural similarities with many Gram-negative heme receptors, and even co-sediments with hemin when expressed in E. coli (293), but is not known to contribute to heme uptake by Neisseria. ZnuD appears to be critical for invasive meningococcal disease, as a N. meningitidis strain lacking ZnuD cannot survive within epithelial cells (294), and is defective for dissemination in a mouse model (292).

As mentioned, TdfF and TdfG are largely uncharacterized, but some information about their regulation at the transcriptional level has been established. Both the tdfF and tdfG genes are repressed in the presence of iron, suggesting a role in iron uptake, and distinguishing them from the zinc-associated (and indeed zinc-repressed) tdfH and tdfJ loci (295, 296). However, to date no iron-binding ligand has been identified for TdfF and TdfG, and little is known about their function, except that TdfF is required for gonococcal survival in human cervical epithelial cell culture (297). This, combined with its iron-repressed phenotype, suggests that its host ligand, if indeed one exists, may be intracellular in nature. TdfF is found in both pathogenic Neisseria species, while TdfG is restricted to only the gonococcus.

C. The Ton Motor Complex

The eight systems described above all depend on the Ton motor complex, which energizes their transport of iron, iron chelates, heme, and zinc (298, 299). The Ton motor is
a tripartite system consisting of three proteins localized in the cytoplasmic membrane:

TonB, ExbB, and ExbD. Recent reports have suggested that ExbB and ExbD complex in a 5:2 stoichiometry, with ExbB creating a pentameric pore surrounding an ExbD dimer (300). Despite this evidence, however, a detailed structure of the full Ton motor is not available, and the precise oligomeric state of TonB within the overall complex is not known. TonB is known to form a dimer *in vivo* (301), but the physiological relevance of this is not understood. Further, TonB is thought to cycle through a repetitive association/dissociation phase with the TdTs (302, 303), but again the importance of this interaction is unknown.

Regarding function, only the broad strokes of Ton motor activity are established. In short, upon ligand docking with one of the TdTs, a conformational change within the transporter exposes the TonB-box, a short N-terminal amino acid sequence, to the periplasmic space. The C-terminal end of the TonB protein, which itself is periplasmic and extends upwards from the cytoplasmic membrane, then forms a stable complex with the TonB-box, thereby physically connecting the TdT to the cytoplasmic membrane (304, 305). This interaction occurs with high affinity, with a $K_d$ in the tens of nanomolar (306). The exact mechanism for TonB-mediated transport after formation of this complex is not fully understood, but several models have been proposed, all of which rely on the complex harnessing the proton motive force to displace or alter the conformation of the TdT plug domain, such as by pulling, rotation, or denaturation (247, 307). For example, in the “pulling” model, the motor uses the C-terminal end of TonB, bound to the TdT plug, to pull the plug towards the periplasm and partially unfold it, thus creating a channel in the TdT β-barrel which is large enough for the bound nutrient(s) to traverse (247). Molecular
dynamics simulations have suggested that such a model is indeed feasible within the constraints of the TonB/TdT binding interaction and proton motive force energy availability (308).

D. ABC Transporters

After passage through the outer membrane, an iron or zinc atom must next be shuttled across the periplasmic space, through the cytoplasmic membrane, and into the cell’s cytoplasm. Primarily, this action is facilitated by dedicated ATP-binding cassette (ABC) transporters, which consist of three parts: a periplasmic binding protein, which binds metal ions on the inner side of the TdT at the outer membrane; a cytoplasmic permease, which forms a pore in the cytoplasm for entry into the cell; and a ATPase, which energizes the system through hydrolysis of ATP (309, 310). Multiple ABC transporters are encoded within the Neisseria genome, and the TdTs are collectively serviced by three such systems. The transferrin and lactoferrin systems utilize the FbpABC transporter (257, 311, 312), FetA uses the FetBCD system (281), and TdfH and TdfJ utilize ZnuABC (249, 313, 314). Elsewhere in the literature, ZnuABC has been called MntABC as a result of apparent interactions with manganese as well as zinc, suggesting a dual function may be present for this system (315, 316). No dedicated ABC transporter for heme is known to exist in the Neisseria, and no known interaction between TdfF and TdfG and any of the ABC transporters has as yet been identified, though it is reasonable to suspect one may exist.

E. Regulation

As with the mammalian systems described earlier, proper maintenance of metal uptake and homeostasis is critical for the Neisseria as well, and as such its iron and zinc
uptake systems are tightly regulated. As a pathogen, it also must respond rapidly to the onset of metal limitation within the host. To that end, the iron uptake systems are globally regulated by the ferric uptake regulator (Fur), and the zinc systems by the zinc uptake regulator (Zur) (296, 317). When iron is replete, Fe$^{3+}$ ions incorporate into dedicated binding sites of Fur monomers, resulting in a conformational change that facilitates formation of a Fur dimer. Dimerized Fur contains DNA-binding motifs which recognize a specific DNA sequence in the promoters of Fur-regulated genes – the so-called “Fur box.” This interaction physically blocks promoter access by RNA polymerase, thus preventing gene transcription. As iron stores are depleted, sufficient Fe$^{3+}$ to maintain Fur in its dimeric state is lost, and repression of transcription is lifted (318-320). Zur functions by the same mechanism in response to zinc excess/limitation, repressing transcription by binding a Zur box in the promoter region (321, 322).

VIII. Research Objectives

The overarching goal of the research described in this dissertation is to characterize the gonococcal zinc importer TdfJ, both from a structure-function perspective and in the context of TdfJ as a potential vaccine antigen. Within this overall scope, we pursued three primary objectives. 1) We identified and performed initial characterization of the interaction between TdfJ and its human ligand S100A7. This work began during my master’s thesis and was published shortly after the beginning of my PhD training, and material absent from my master’s thesis is included in this document. 2) We investigated the role of the α-helix finger in extracellular loop three of TdfJ with respect to S100A7 binding and zinc piracy. 3) We utilized TdfJ, both as whole recombinant protein and as a chimeric antigen, in
mouse immunizations to evaluate protection against gonococcal colonization. We also evaluated functional properties of the antibody response generated by TdfJ-based vaccination.
CHAPTER 2: RECENT PROGRESS TOWARDS A GONOCOCCAL VACCINE

I. Introduction

The idea of immunizing against gonococcal disease is not a recent development, and indeed dates to shortly after the identification of *N. gonorrhoeae* as the etiological agent of the disease. Despite interest in such a vaccine, the early efforts were unsuccessful. While gonococcal infections have largely been controllable since the introduction of antibiotics in the early 20th century, continued accrual of antimicrobial resistance mechanisms and the emergence of highly-drug-resistant isolates has resulted in a renewal of vaccine development efforts (74, 323). Vaccination failures have largely been attributed to three major factors: 1) Gonococcal infection does not confer a protective immune response in humans, and therefore the lack of an observable immunity to infection has made it impossible to establish correlates of protection for the disease (324). 2) *N. gonorrhoeae* is known to modulate the antigenic presentation of many of its main surface structures with high frequency, complicating the evaluation of any individual target as a suitable immunogen. Beyond this, many of these antigens share characteristics with those found in the commensal *Neisseria* species, and therefore many adults possess anti-gonococcal antibodies regardless of exposure to the pathogen, making identification of potentially protective antigens difficult (325). 3) Gonorrhea is a human-restricted disease, which severely limits the utility of animal models. Effectively replicating the human-like disease state has proven to be a large obstacle in evaluating immune responses and candidate vaccines. This chapter will provide a short review of recent progress towards a viable gonococcal vaccine, with a major focus on antigen identification and characterization, as
well as computational tools which have aided this process. It will also touch on aspects of animal modeling and immune responses to gonorrhea.

II. Previous Vaccine Efforts

Previous attempts to generate an effective gonococcal vaccine have been unsuccessful. As mentioned, the propensity for *N. gonorrhoeae* to alter the antigenic identity and expression levels of surface structures has made identification of vaccine targets challenging. To date only two candidate gonococcal vaccines have reached human trials. One candidate targeted pilin, the major component of the type IV pilus (326), though no difference in protection was seen between placebo and vaccinated groups. It is likely that antigenic variability of pilin contributed to protection failures in this trial (327). The other trial utilized heat-killed, partially lysed whole gonococcal cells that, while able to generate bactericidal antibodies in a high percentage of recipients, did not show long-term protection; within one year post-vaccination, similar numbers of subjects were infected with *N. gonorrhoeae* regardless of their vaccination status (328, 329). Despite these failures, other immunization efforts have shown that it is at least possible to reduce susceptibility to gonococcal infection in chimpanzees (330). This study suggested that natural infection generates some level of acquired immunity in cases of gonococcal urethritis or pharyngitis, as previously colonized chimps required a significantly higher bacterial dose to achieve reinfection. However, protection waned over time, and immunity was not seen when previously “immune” chimps were reinfected years later. Finally, PorB has received considerable attention as a vaccine antigen due to its roles in adherence and dissemination. For example, Zhu *et al.* formulated with PorB as a DNA-based vaccine (331), which
expressed porB from plasmid, and as recombinant protein alone or presented in viral replicon particles (332). The DNA-based vaccine was able to induce both a Th1 and Th2 response, depending on method of delivery, and the viral replicon particle-based method showed some protective capability in mice, but ultimately wasn’t pursued further. Prior to this, an animal trial was conducted comparing porin formulated in liposomes, proteosomes, and gonococcal membrane blebs (333). This study found that porin delivered in liposomes, which mimics the in vivo structure, generated greater numbers of anti-porin antibodies, including those that recognized surface-exposed sections of porin, compared to other delivery methods. However, this study did not proceed to clinical trials.

III. Recent Antigen Characterization

A. TonB-dependent Transporters

The gonococcal TdTs, as previously described, play essential roles in establishing disease by virtue of their nutrient uptake mechanisms. Beyond this, however, the TdTs have received appreciable attention as vaccine targets due to their overall high levels of conservation among gonococcal isolates, and their limited propensity for high-frequency variation. The TdTs are in many cases capable of eliciting an antibody response (291, 334), but questions abound over the correlation between antibody levels and protection. Further, it is feasible that the binding of host ligands during metal piracy may generate a quasi-mimicry of self-antigens, thus contributing to immune evasion. Interestingly, however, Frandoloso et al. showed that a nonbinding mutant of the surface lipoprotein TbpB in Haemophilus parasuis, which cannot interact with transferrin but still retains the overall structure of the wildtype, can generate an enhanced protective response compared to
native TbpB in a porcine challenge model (335). While it is unclear whether this phenomenon is transferable to the transporters themselves, Cash et al. found that mutagenesis of the gonococcal TdT TbpA could abrogate human transferrin binding without appreciable disruption of the overall TbpA structure (254). As generation of a nonbinding gonococcal TbpA appears possible, using it as an immunogen to test protection relative to the wildtype may be a promising way forward.

Despite these advantages, however, large-scale production of integral transmembrane proteins such as the TdTTs presents a few technical limitations that may impact commercial vaccine endeavors. 1) Such proteins are produced at relatively low levels in membranes, which necessarily slows production times. 2) Stabilization and solubilization of these proteins require highly-optimized detergent concentrations. 3) Purification directly from membranes requires specific expertise and equipment that is not necessary for purification from the cytoplasm. To address these issues, Fegan et al. (336) utilized an innovative approach to generate a TbpA/B hybrid antigen. This novel antigen utilizes the C-lobe of the immunogenic lipoprotein TbpB as a soluble protein “scaffold” onto which putatively immunogenic extracellular loop sections of TbpA are transplanted in place of TbpB loops. Antisera raised against these hybrid antigens recognized surface-exposed TbpA of N. meningitidis and was capable of inhibiting transferrin utilization by gonococci.

Previously, Price et al. generated a similar hybrid by fusing gonococcal TbpA loop 2 to the N-terminal lobe of TbpB, using the A2 domain of cholera toxin as a hosting molecule. These chimeras generated bactericidal and growth-inhibitory antibodies against N. gonorrhoeae (337).
B. Lipooligosaccharide

Gonococcal LOS has garnered attention recently as a feasible vaccine candidate (338). While LOS is subject to phase variation (339), it is easily accessible on the gonococcal surface, where it is present in abundance. Furthermore, while many LOS antigens share structures with human glycosphingolipids thus making them ineligible for targeting, two distinct LOS epitopes, recognized by monoclonal antibodies L8 and 2C7 (340, 341), do not suffer this pitfall. A recent study (342) showed that the 2C7 epitope, which is widely conserved amongst gonococci, is immunogenic during natural infection. As such, the 2C7 epitope has attracted attention as a vaccine target. Due to the complications of purifying or directly synthesizing oligosaccharide structures, along with their generally poor immunogenicity, an attractive prospect for utilizing such antigens is to create a peptide mimic – a so-called “mimitope” – with a highly similar structure to the natural oligosaccharide. For 2C7, this was successfully done in 2006 (343), and continuing work showed that immunization with this mimitope alongside a Th1-stimulating adjuvant resulted in generation of bactericidal IgG, reduced gonococcal colonization, and sped bacterial clearance in experimentally infected mice (344). As LOS plays a key role in the gonococcal pathogenic lifestyle and a 2C7-based vaccine would ostensibly offer broad cross-reactivity against gonococcal strains, this represents an important step forward in the quest for gonococcal prevention.

C. Meningococcal Vaccines and Outer-membrane Vesicles

Vaccines against *N. meningitidis* serogroup B have been suggested to confer protection against gonococcal infections in addition to their intended meningococcal role.
Various observational studies noted a decrease in gonococcal infections in regions after administration of meningococcal group B outer-membrane vesicle (OMV) vaccines (345-347), perhaps owing to the high level of genetic similarity between *N. meningitidis* and *N. gonorrhoeae*. In a case-control study of the meningococcal group B OMV vaccine administered in New Zealand, MeNZB (348), researchers estimated the vaccine to be approximately 31% effective for gonococcal cross-protection, with effectiveness waning over time (349). Bioinformatic analyses have compared MeNZB, which is no longer on the market, to the currently-available meningococcal group B vaccine 4CMenB (GSK), which contains the MeNZB OMV antigen alongside *Neisseria* adhesin A (NadA), factor H binding protein (fHbp), and *Neisseria* heparin binding antigen (NHBA) (350). These antigens were recently compared to their homologous gonococcal proteins, and assessed for their ability to generate anti-gonococcal antibodies (351). These analyses found that *N. gonorrhoeae* strain FA1090 encodes homologues to 20 of 22 core proteins from the Bexsero OMV, of which 16 share >90% identity with the vaccine antigen. While the *nadA* gene is absent and fHbp is not believed to surface exposed in the gonococcus, NHBA from FA1090 is approximately 69% identical to the 4CMenB variant. Furthermore, this study showed that OMV-derived antibodies can recognize gonococcal proteins, and others demonstrated that immunization with 4CMenB accelerates gonococcal clearance in a mouse challenge model (352). The exact nature of cellular immunity following 4CMenB vaccination is not clear, but a recent study identified upregulation of IFN-γ, IL-4, and IL-10 upon OMV introduction, suggesting some capacity for T-cell stimulation (353).
OMVs are bi-layered membrane spheres that are naturally released from Gram-negative bacteria, including the *Neisseria*, and have received considerable attention as vaccine antigens for STI pathogens (354). Their surface is decorated with phospholipids, various outer-membrane proteins, and lipopolysaccharide/lipoooligosaccharide (355). Many common OMV proteins in addition to those discussed above share similarity between *N. gonorrhoeae* and *N. meningitidis* (356), making OMVs a promising platform for a universal pathogenic *Neisseria* vaccine. For the gonococcus, an OMV preparation with microencapsulated IL-12 as an adjuvant was recently shown to accelerate gonococcal clearance and to induce *N. gonorrhoeae*-specific antibodies when administered intravaginally to BALB/c mice (357). Critically, this combination conferred protection against heterologous gonococcal strains (358), which is necessary for effective vaccination. In another study, an OMV from *N. meningitidis* that was modified to overexpress a mutant fHbp and an attenuated endotoxin was able to elicit human complement-mediated serum bactericidal antibodies against *N. gonorrhoeae* (359). More recently, detoxified meningococcal OMVs as immunogens demonstrated a protective effect against gonococcal challenge in mice, which was associated with anti-OMV immunoglobulins that cross-reacted with the gonococcus (360).

**D. NHBA and Bacterial Ghosts**

The previous section briefly discussed NHBA as a component of the 4CMenB vaccine, but this antigen has also independently received attention as a contributor to protection against gonococci. Gonococcal NHBA is surface exposed and is involved in interactions with host cells (351). Furthermore, this antigen is conserved across gonococcal genomes and has
a high sequence identity among isolates. It was recently shown that recombinant NHBA is immunogenic and that anti-NHBA antibodies recognize a wide range of gonococcal NHBA variants (361). In addition, these antibodies promote C3 deposition and opsonophagocytosis. The biological importance of NHBA should not be overlooked, as it plays a role in formation of gonococcal microcolonies and adherence to epithelial cells (362).

An interesting adjunct to vaccination platforms is that of the bacterial ghost. Ghosts are empty shells of Gram-negative bacteria produced from the expression of bacteriophage PhiX174 lysis gene E (363). Expression of gene E in non-host-range bacteria for PhiX174 leads to conversion of Gram-negative bacteria into ghosts, rather than causing lysis. Critically, these ghosts expel their nucleic acids, cytoplasmic proteins, and ribosomes during formation, leaving behind a fused outer and inner membrane with an antigenic profile identical to that of the counterpart bacteria. As a result, these ghosts can be utilized for immunization efforts involving envelope antigens, and can even be adapted as delivery vehicles for molecules such as soluble protein or DNA (364). Jiao et al. (365) recently generated a *Salmonella enteriditis* ghost for the delivery of a PorB-based *N. gonorrhoeae* DNA vaccine. In this study, the eukaryotic expression plasmid pVAX1 containing a full-length *porB* gene was loaded into ghosts and used to transfect mouse bone marrow-derived dendritic cells (BMDCs), which were activated in response to the ghost. In addition, mice produced PorB-specific antibodies upon immunization, and mice immunized with the PorB ghost showed elevated CD4⁺ and CD8⁺ T-cell counts compared to those immunized with ghost alone, and both groups were higher than the control group. In a later study (366), this
S. enteriditis ghost was again utilized for the delivery of a gonococcal nspA-based vaccine. The nspA gene was cloned into plasmid and loaded into the ghost, which was used to immunize BALB/c mice either alone, or alongside ghosts containing the gonococcal porB gene, as previous reports showed PorB to be an effective adjuvant for this system when delivering S. enteriditis antigens (367). When co-administered, these ghosts elicited specific IgG and stimulated lymphocyte proliferation. Therefore, the bacterial ghost delivery method may be a promising approach for future gonorrhea vaccine studies.

E. Epitope Prediction Tools

Recently, high-throughput computational methods have been utilized to speed the process of epitope prediction and characterization for vaccine development. These techniques have even been used to design synthetic particles that themselves may act as vaccine antigens. For example, Jain et al. (368) utilized these methods to establish a functional pipeline for identifying possible N. gonorrhoeae epitopes. In short, the SignalP server (DTU Health Tech), which utilizes artificial networks and known organism proteomic databases to predict the presence and location of signal sequences and cleavage sites, was used to predict secretory proteins from the gonococcus. Candidates from this screen were then submitted to HMMTOP (369), a tool for membrane topology prediction, for validation. Finally, candidate proteins were analyzed by VaxiJen 2.0 for antigen prediction (370) and by HLAPred for identification of T- and B-cell epitopes. This pipeline resulted in identification of 23 potential gonorrhea vaccine candidates for utilization in further characterizations.

PubMLST contains a large database of N. gonorrhoeae genomic data. Baarda et al. (371) outlined a methodology for “mining” these alleles from PubMLST which may be
applied to eventual vaccine design. In this method, one can query a given gonococcal gene and find all alleles of the gene, including their translated protein sequence, within the database. From this point, one can see sequence conservation and distribution within the population, and then proceed to species-specific polymorphism analysis. At this stage, the user can map polymorphisms onto confirmed protein structures via 3D protein analysis tools like PyMOL or UCSF Chimera and identify the conservation of specific antigenic targets, if any are known. In a test example of the workflow, Baarda et al. examined 3,908 *N. gonorrhoeae* genomes to identify polymorphisms in the MtrE protein. Cumulatively they found that five variants of MtrE represent 98% of all gonococcal MtrE diversity, with a large percentage of the protein being conserved across all isolates. Moreover, structural mapping for MtrE revealed the locations of low- and high-prevalence polymorphisms within the protein. Such analyses for MtrE and other gonococcal proteins are a useful step forward in targeted, rational vaccine design based on exposed, conserved epitopes.

MtrE has been included in other computational vaccine studies as well, such as that performed by Wang et al. (372), which utilized ferritin nanoparticles as a scaffold for antigenic MtrE loops. The self-assembling ferritin can house relatively large protein domains on its N-terminus (373), so Wang et al. utilized a non-human ferritin to present MtrE surface-exposed loops as vaccine antigens. These efforts showed that the chimeric molecule could assemble and present loops in a way that should allow antibody access. During the design phase of these chimeras, structural data from the MtrE crystal’s antigenic loops was copied and inserted into a model of the ferritin cage, then the preliminary chimera models were refined for thermodynamic favorability in 3Drefine (374). These chimeras were then
purified, and their crystal structures were solved, showing that insertion of MtrE loop peptides did not disrupt the overall structure of the ferritin cage, suggesting that this nanoparticle method could be a suitable platform for delivering \textit{N. gonorrhoeae} immunogens alone or in an ordered array.

\section*{IV. Animal Models}

As discussed, gonorrhea is a human-specific disease, and this limitation has created difficulty in animal modeling of the infection. Early efforts were successful only in chimpanzees, which develop a gonorrhea-like infection, but their use ultimately proved too costly to be practical (330). Today, a common model of infection is the 17 β-estradiol mouse model, which arose after the discovery that the mouse estrous cycle affects gonococcal colonization (375, 376). The exact mechanism by which estradiol treatment facilitates colonization is not known. Current hypotheses include the mediation of immune cell function by sex hormones (377), estrous cycle-dependent variation of innate immunity mediators (378), and rearrangements of genital tissue (379). While this model is still quite limited, it allows for colonization experiments of about 2 weeks in duration, which has proven useful in initial characterization of vaccine candidates (380). Indeed, there are broad similarities between the murine and human vagina, including carbon availability, mucus properties, and commensal burden (381, 382). However, it is notable that that mouse vaginal pH is considerably higher than that of the human, and murine iron sources such as transferrin and lactoferrin are not useable by the gonococcus, which only recognizes the human forms of these proteins (383). Numerous attempts at humanizing mice to strengthen their utility as an infection model have been made, and these efforts continue
today. Most of these have been targeted at cell surface receptors, complement regulators, and metal sequestering proteins. To date, transgenic mice capable of expressing human CEACAMs (384), human factor H (385), and transferrin (386) have been successfully bred, with numerous others currently in development.

V. Immune Responses to Gonorrhea

The mouse model has also been instrumental in monitoring the immune response to gonorrhea. As mentioned before, no protective response is generated upon gonococcal colonization, and indeed *N. gonorrhoeae* is known to specifically dampen the adaptive arm of the immune system (387). Experimental mouse infections have demonstrated that gonococcal infections stimulate production of cytokines such as IL-17 and IL-22, but inhibit production of others such as IL-4 or IFN-γ, suggesting a pathogen-driven bias towards a Th17-mediated innate-like response and suppression of Th1 and Th2 (388-390). Such a response is consistent with known observations regarding gonococcal disease, specifically a large neutrophil influx. Other studies have identified TGF-β as an important mediator of this dichotomous cellular response; administration of anti-TGF-β antibodies during infection is associated production of gonococcus-specific antibodies and stimulation of IL-4- and IFN-γ-secreting cells (107, 391). Cumulatively, this response led to immune memory and accelerated clearance, corroborated by evidence which showed that reinfection of the antibody-treated mice demonstrated enhanced resistance. In a similar vein, mouse studies also revealed an upregulation of IL-10 during infection (106), and subsequent observations using IL-10-deficient mice or neutralizing anti-IL-10 antibodies showed the development of a protective antibody response. It is noteworthy that these observations are not exclusive
to the mouse model, as human gonorrhea patients show elevated IL-17 and IL-23 as well (392). When considered as a whole, these data would suggest *N. gonorrhoeae* selectively upregulates innate-like immune responses, and indeed the organism seems capable of resisting such defenses. For example, as described in chapter 1, gonococcal TdfH facilitates resistance to neutrophil NETs, and IL-17 is a potent chemoattractant for said neutrophils (393).

**VI. Concluding Remarks**

*Neisseria gonorrhoeae* has presented challenges to researchers for decades. This pathogen causes illness in millions of humans and utilizes an arsenal of antimicrobial resistance strategies and highly-variable surface structures to make treatment and prevention an enormous challenge. Despite these difficulties, the studies described above represent promising steps forward in the effort to combat this urgent threat pathogen. Bioinformatic tools and computational methods now make antigen identification and characterization easier than ever, so it is possible that new targets will emerge as well. This may prove critical to our efforts in combating a pathogen with super bug potential.

Author’s Note: Chapter 2 contains text sections from Maurakis, S. A., & Cornelissen, C. N. (2022). Recent Progress Towards a Gonococcal Vaccine. *Frontiers in Cellular and Infection Microbiology, 12*. Doi:10.3389/fcimb.2022.881392 (394). All Frontiers content is published under a CC-BY Creative Commons attribution license, which enables anyone to use the publication freely, given appropriate attribution to the author(s) and citing Frontiers as the original publisher.
CHAPTER 3: GONOCOCCAL TDFJ INTERACTS WITH S100A7

I. Introduction

TdfJ is one of eight TonB-dependent transporters produced by the *Neisseria*. Early characterization of gonococcal TdfJ, and its meningococcal homologue ZnuD, revealed these proteins as key contributors to zinc uptake in zinc-limited conditions (287, 291). However, unlike the other zinc importer TdfH (CpbA in *N. meningitidis*), which utilizes zinc from calprotectin, no ligand for TdfJ was described. Calprotectin is a member of the S100 calcium binding protein family, of which there are currently 24 identified members divided according to their primary location of function at the cellular level: intracellular only, extracellular only, or both (395). They are differentially expressed according to tissue type, and many are capable of restricting bacterial growth through metal ion sequestration (396). S100A7, also called psoriasin due to its upregulation in psoriatic lesions (218, 397), has demonstrated zinc-mediated antimicrobial activity against *S. aureus*, *Pseudomonas aeruginosa*, and *E. coli* (219, 240, 241, 396, 398, 399). S100A12 (Calgranulin C) has demonstrated similar effects against several bacteria and parasites (227, 233, 396, 400). Critically, the S100s share similar structural characteristics, and the known interaction between TdfH and calprotectin makes the other zinc-binding S100s a promising target for identifying a potential TdfJ ligand.

Herein, we describe the conservation, regulation, and phenotypic characterization of TdfJ. TdfJ has been described as having differential responses to the presence of iron and zinc (287), and in this study, we demonstrate that it shows additive repression by zinc and induction by iron. Furthermore, we show that wild-type *N. gonorrhoeae* is capable of
utilizing zinc bound to S100A7 and S100A12 as a sole zinc source, and that utilization of S100A7 occurs in a TdfJ-dependent manner. Finally, we show that S100A7-mediated zinc restriction induces tdfJ gene expression in an equivalent manner to that seen upon addition of a zinc-specific chelator. This study marks the first time that S100A7 has been posited to be directly utilized as a zinc source by an invading pathogen.

II. Results

A. The tdfJ gene is found in all Neisseria species and is highly conserved

We evaluated the predicted amino acid sequence identity among the TdfJ proteins or their homologues in Neisseria genomes available in NCBI databases, and found that the protein is highly identical across all queried isolates. To demonstrate this, we performed multiple alignment of 50 of these sequences in Geneious® (Biomatters, Ltd.) and found that the proteins shared 96.2% pairwise identity. Furthermore, 82.9% of amino acid residues in the proteins were identical across all sequences. It should be noted that a few sequences selected contain a six-residue stretch (MRREAK) at the amino terminus, as there is some database inconsistency as to which of two close-proximity methionines is the true start of the protein. For the purposes of alignment, these residues were ignored, but all six are identical in the sequences which include them. We also generated a consensus sequence from those queried and submitted it to DTU Bioinformatics NetSurfP-2.0 (http://www.cbs.dtu.dk/services/NetSurfP/) for domain prediction, which identified putative β strands that constitute the protein’s transmembrane domains. Using the consensus sequence and predicted β barrel, we generated a topology prediction for TdfJ using TOPO2 from the University of California at San Francisco
The resulting model was adapted to show the conserved and variable residues of TdfJ within the 2D topology diagram (Figure 3).

B. Gonococcal TdfJ is zinc repressed and iron induced

We next identified the conditions under which TdfJ was maximally produced in *N. gonorrhoeae* wild-type strain FA19 (401). We previously reported that strain FA1090 shows decreased TdfJ production in the presence of zinc and increased production in the presence of iron (287), but the additive effects of these two metals in strain FA19 were not assessed. We performed western analysis of whole-cell lysates prepared from strain FA19 grown in Chelex-treated chemically defined media (CDM) supplemented with zinc, iron, and/or the high affinity zinc chelator *N*,*N*,*N′*,*N′*-tetrakis-(2-pyridinylmethyl)-1,2-ethanediamine (TPEN) at the concentrations indicated. We found that levels of TdfJ were decreased in the presence of zinc and increased in zinc-deplete conditions (Figure 4A). TdfJ production was almost nonexistent when zinc was present and iron absent, which agrees with the observed phenotype for FA1090 (287). When both zinc-deplete and iron-replete conditions were established, we observed an additive effect of their respective regulation inputs. Equal sample loading was verified by Ponceau stain of the protein blot. We then performed densitometry analysis on N = 3 blots and verified that zinc-restricted bacteria produce significantly more TdfJ than those grown in zinc-replete conditions, and that when zinc is scarce, TdfJ production is significantly higher when iron is present than when it is absent (Figure 4B). These results established for us a set of conditions that are optimal for TdfJ production, and these were kept consistent in subsequent experiments.
Figure 3. Predicted topology and conservation of TdfJ and its homologues

Amino acid sequences for 50 examples of gonococcal TdfJ or its homologue in *N. meningitidis*, *N. lactamica*, or *N. elongata* (accession numbers are available in the Materials and Methods) were aligned using Geneious and submitted to NetSurfP-2.0 for prediction of transmembrane domains and intra-/extracellular loops. This information was used to create a topology prediction in TOPO2, which was then adapted to create the above model, which has the protein’s cleavable N-terminal signal sequence removed. The two horizontal lines represent the outer membrane, and each amino acid residue of the protein is represented by a circle. Residues in white are identical across all 50 sequences, while those in red have 1 or more mismatches, and the most common residue is indicated. Residues outlined in blue make up β sheets. Two residues are shown in green. These two residues are variable, and 9 of the 50 sequences queried contain a conserved KEEG motif between these two residues. Figure from (249).
Figure 4. TdfJ is zinc repressed and iron induced

(A) Strain FA19 was grown in CDM until exponential growth, then back diluted in the same media and treated with Fe(NO$_3$)$_3$, ZnSO$_4$, and/or TPEN in the concentrations indicated. These cultures were grown for 6 hours before whole-cell lysates of equalized density were prepared and subjected to SDS-PAGE and subsequent immunoblotting with antiserum raised against TdfJ. The Ponceau stained blot shows equal protein loading. Blot is representative of N = 4 experiments. (B) Western blots shown in panel (A) were analyzed for signal intensity using BioRad Image Lab software. Statistical significance was determined using an unpaired Welch’s t-test. *, P ≤ .05; **, P ≤ .005; ***, P ≤ .0005; ****, P ≤ .0001. Figure from (249).
C. Wild-type gonococci can utilize zinc bound to S100A7 and S100A12 in vitro

Considering the regulation of TdfJ by zinc, we hypothesized that TdfJ contributes to zinc acquisition by the gonococcus, and that the zinc-bearing ligand for TdfJ belongs to the S100 family of proteins. To test this hypothesis, we assayed the ability of wild-type strain FA19 to grow in the presence of S100 proteins that are reported to bind zinc. For these studies the dimeric S100 proteins were loaded to ~25% saturation with zinc in zinc-deplete conditions. Strikingly, we found that S100A7 and S100A12, which have antimicrobial activity against other pathogens (396), were able to support growth of the gonococcus as a sole zinc source in a manner similar to that observed in the presence of ZnSO$_4$ alone (Figure 5). When no zinc source was provided, the gonococci ceased to grow after approximately 2 hours. We hypothesize that the bacteria’s internal zinc stores could sustain it for roughly one doubling before no longer being sufficient. These data imply that *N. gonorrhoeae* is able to overcome host nutritional immunity by directly co-opting host zinc-sequestering proteins S100A7 and S100A12, similar to Calprotectin as we previously observed (287).

D. TdfJ is necessary for gonococci to utilize S100A7 as a zinc source

We grew a gonococcal mutant, strain MCV928 (402), which is incapable of producing TdfJ, in zinc-depleted conditions containing S100A7 and assessed growth over 6 hours. In the absence of TdfJ, S100A7 was completely unable to support the growth of *N. gonorrhoeae* as a zinc source (Figure 6). Indeed, these samples were statistically indistinguishable from those containing no zinc. S100A12 fully supported growth of the *tdfJ* mutant strain, MCV928, indicating that its use as a zinc source is unrelated to TdfJ. Considering the fact that utilization of S100A7 depends on TdfJ presence, we analyzed
Figure 5. *Neisseria gonorrhoeae* strain FA19 utilizes S100A7 and S100A12 as a sole zinc source

Wild-type *N. gonorrhoeae* strain FA19 was grown in Zn-limited conditions in defined media (CDM), which was supplemented with growth premix containing either ZnSO₄, S100A7, S100A12, or no zinc. Growth was measured by OD₆₀₀ readings every 2 h for 6 h total. The graph demonstrates means and SD for N = 3 independent experiments. A 2-way repeated measures ANOVA with Tukey’s correction was performed for all means, with significance at 6 h shown. *, P ≤ .05; **, P ≤ .005; ***, P ≤ .0005; ****, P ≤ .0001. Figure from (249).
Figure 6. *Neisseria gonorrhoeae* requires TdfJ to use S100A7 as a zinc source

The *N. gonorrhoeae* *tdfJ* null mutant, MCV928, was grown in Zn-restricted CDM supplemented with growth premix containing ZnSO$_4$, S100A7, S100A12, or no zinc. Growth was measured by OD$_{600}$ readings every 2 h for 6 h total. Graph is representative of N=3 experiments and shows mean and SD. A 2-way repeated measures ANOVA with Tukey’s correction was performed for all means, with significance at 6 h shown. *, P ≤ .05; **, P ≤ .005; ***, P ≤ .0005; ****, P ≤ .0001. Adapted from (249).
whether its presence altered the expression of the *tdfJ* gene, as this would be the expected outcome for a zinc-sequestering protein affecting a zinc-responsive gene. To test this, we grew strain FA19 in CDM supplemented with Fe(NO₃)₃ and equal amounts of either TPEN or apo-S100A7 to induce zinc stress. Western analysis of these samples indicated that zinc stress imposed by S100A7 results in TdfJ protein levels that are indistinguishable from those induced by TPEN presence (Figure 7). Cumulatively, these data suggest S100A7 as the human zinc-bearing ligand for TdfJ.

### III. Discussion

Nutritional immunity, the sequestration of essential nutrients by hosts to hamper invading pathogens, and the strategies deployed by bacteria to overcome this defense, has become a prevalent theme in the evolving host-pathogen struggle. Iron, zinc, manganese, and other transition metals are key nutrients for bacterial survival and thus infection (153, 403). *N. gonorrhoeae* is particularly capable of overcoming nutritional immunity using non-traditional mechanisms. Unlike many other pathogens, *N. gonorrhoeae* does not produce any siderophores and therefore does not directly scavenge free iron from its environment. Instead, it utilizes an arsenal of TonB-dependent transporters that bind directly to host nutritional immunity factors including transferrin, lactoferrin, and hemoglobin and strip them of their iron cargo, effectively co-opting them for support of infection (404). More recently, it has become clear that this phenomenon is not restricted to only iron, as both the gonococcus and meningococcus can use other TdTs to acquire zinc by a similar strategy (286, 287, 291). Considering the high level of structural and functional conservation of the gonococcal TdTs, we hypothesized that the uncharacterized TdTs likewise play an important
Figure 7. S100A7 presence induces production of TdfJ

Strain FA19 was grown in CDM until exponential growth, then back diluted in the same media and treated with Fe(NO$_3$)$_3$, and either TPEN or S100A7 in the concentrations indicated. These cultures were grown for 6 hours before whole-cell lysates of equalized density were prepared and subjected to SDS-PAGE and western blotting to detect TdfJ. Equal protein loading is demonstrated by Ponceau staining. N = 3 represented. Figure from (249).
role in the ability of *N. gonorrhoeae* to survive and grow in the context of human nutritional immunity factors. Critically, recent studies have shown these transporters to be promising targets for vaccine development (254, 335).

The S100 proteins play several essential roles in vertebrates, including sequestration of transition metals to starve invading bacteria of key nutrients (396). Calprotectin, S100A12, and S100A7 have been shown to exert suppressive activity against numerous pathogens, including *E. coli*, *S. aureus*, *P. aeruginosa*, *Shigella flexneri*, *Helicobacter pylori*, and *C. albicans*. This report and our previous work (287) demonstrate that *N. gonorrhoeae* is able to overcome nutritional immunity by utilizing these otherwise antimicrobial proteins as zinc sources. Jean et al (287) showed that *N. gonorrhoeae* utilizes TdfH for binding calprotectin and consequent acquisition of zinc. Moreover, the meningococcal TdfJ homologue, ZnuD, has demonstrated binding of extracellular zinc (292), and both ZnuD and TdfJ are regulated by zinc via the regulator Zur (296), which is consistent with the expected phenotype of zinc importers. TdfH and ZnuD have been shown to contribute to *Neisseria* growth in zinc-deplete conditions (286, 287, 291). For these reasons, we sought to evaluate S100A12 and S100A7 as potential ligands for other TdTs, specifically TdfJ. Indeed, S100A7 can only serve as a sole zinc source when *N. gonorrhoeae* produces a functional TdfJ. In *vitro*, this interaction, like the other TdT-mediated interactions, allows the gonococcus to overcome metal sequestration and grow effectively.

Our finding that TdfJ is both induced by iron and repressed by zinc agrees with previous studies, and here we showed that these two regulators have additive effects on gene expression. When considered independently, downregulation by zinc had a slightly
more pronounced effect than induction by iron, which is consistent with the expected phenotype of a zinc transporter. It is not expected that TPEN presence played any role in unintended chelation of iron from samples where both were present. Among the gonococcal Tdfs, only TdfJ is induced by iron; TdfH is not regulated by iron (287), and two other proteins TdfF and TdfG are in fact iron repressed (262, 297). Despite the apparent regulation of TdfJ by iron, our experiments revealed no clear functional relationship between the two, so the reason for this coordinate regulation is left to speculation. One possible explanation is that TdfJ is the preferred mediator of zinc uptake for *N. gonorrhoeae* when iron is replete; the *tdfJ* gene is found across all of genus *Neisseria* and its protein products are highly conserved. Unlike TdfH, TdfJ is able to bind directly to free zinc, which is then internalized (292). Furthermore, these observations are substantiated by key regulatory motifs in the *N. gonorrhoeae* genome. Pawlik *et al.* (296) identified the sequence of a putative Zur box for meningococci, and we found via pattern location software (http://www.cmbl.uga.edu/software/patloc.html), that a 100% match for this motif (TGTTATATAATAACA) is located within the promoter region of *tdfJ*. We used the same software to search for putative Fur boxes upstream of *tdfJ* and none were identified within 1.5 kilobases from the start of the gene. This agrees with the observation that iron presence enhanced TdfJ production rather than repressing it, as would be expected for the typical function of Fur. Paradoxically, previous reports have shown that the Fur protein binds within the promoter region of *tdfJ*, but the precise binding site was not mapped (320). Therefore, at this time the mechanism of iron regulation of *tdfJ* is unresolved, but has been observed to occur at the transcriptional level (295).
In this chapter, we defined a novel interaction between TdfJ and the human zinc-binding protein S100A7, wherein TdfJ facilitates zinc acquisition for the gonococcus, thus overcoming one type of host-mediated nutrient restriction. This interaction may provide a clear selective advantage to *N. gonorrhoeae* in the context of infection, especially at the mucosal epithelium, a biologically relevant niche for the gonococcus where S100A7 is enriched (240, 241). The S100A15 protein is highly similar to S100A7 and has also demonstrated metal-sequestering antimicrobial effects (238), so it is possible that in these or other host tissues, it may function alongside or in concert with S100A7, but its relationship to *N. gonorrhoeae* and TdfJ was not explored in the current study. We also show that another zinc-binding protein, S100A12, supports gonococcal growth as a sole zinc source by an as-yet-uncharacterized manner. While the mechanism for S100A12 utilization is not yet clear, TonB-independent siderophore-iron uptake has been observed (402, 405). In light of these findings, we suggest that TdfJ is a promising vaccine target for this important pathogen, and we also note the fact that ZnuD has received consideration for the same purpose in meningococcus (406). TdfJ is exposed on the bacterial surface and is not subject to the high-frequency phase and antigenic variation that has disqualified so many other surface structures from consideration. TdfJ may contribute to *in vivo* survival of the gonococcus by utilization of S100A7, which is highly upregulated in the epithelia, and indeed ZnuD has been shown to participate in interactions with epithelial cells (294). Finally, TdfJ is ubiquitously produced across the *Neisseria* species, including the commensals from which gonococci and meningococci frequently acquire resistance factors, and which themselves may be opportunistic pathogens (11). To summarize, we report a novel
interaction between \textit{N. gonorrhoeae} and its human host, which allows the gonococcus to overcome the innate immune mechanism of nutrient starvation. Considering the potential importance of this strategy for infection, alongside TdfJ’s presumed potential as a promising vaccine target, further characterization of the TdfJ-S100A7 interaction is suggested in order to clarify the importance of TdTs as virulence factors for \textit{N. gonorrhoeae}.

Author’s Note: Chapter 3 contains text sections and figures from Maurakis, S., Keller, K., Maxwell, C. N., Pereira, K., Chazin, W. J., Criss, A. K., & Cornelissen, C. N. (2019). The novel interaction between \textit{Neisseria gonorrhoeae} TdfJ and human S100A7 allows gonococci to subvert host zinc restriction. PloS Pathog, 15(8), e1007937. Doi:10.1371/journal.ppat.1007937 (249). All PLOS articles are published under a Creative Commons Attribution license (CC BY), and are available for anyone to download, re-use, reprint, modify, distribute, and/or copy so long as the original authors and source are cited.
CHAPTER 4: MUTAGENESIS OF THE LOOP 3 α-HELIX OF TDFJ INHIBITS S100A7 BINDING AND UTILIZATION

I. Introduction

In the previous chapter, we showed that TdfJ recognizes the human innate immunity protein S100A7 and enables zinc extraction by the gonococcus (249). This interaction is the first of its kind reported for S100A7, which typically exhibits an inhibitory effect on bacterial growth by virtue of its zinc sequestration capabilities (219, 240, 241, 396, 398) and in some cases even shows contact-dependent killing of microbes (239). The *N. meningitidis* homologue of TdfJ, ZnuD, is nearly identical (>97%) to TdfJ in amino acid sequence. The crystal structure of ZnuD (PDB: 4RDR and 4RDT) shows that extracellular loop three contains two zinc-sensing regions, which are enriched in histidine, aspartate, and glutamate. Furthermore, this region undergoes considerable rearrangement in a substrate-dependent manner (Figure 8). When zinc is absent, the region of loop three between the zinc-sensing clusters adopts an α-helical configuration that extends far from the TdT barrel. Conversely, in the presence of zinc, this region is remodeled into a pair of flexible β-strands, exposing zinc in the peripheral binding site to a newly available, high-affinity site buried deeper in the barrel (292). While such rearrangement has been observed in other zinc binding proteins (407-409), this is a novel phenomenon among the TdTs. In another study, Cash *et al.* (254) demonstrated that an α-helix in loop three of another TdT, TbpA (Figure 9), plays a vital role in interaction with, and subsequent iron extraction from, human transferrin. Therefore, we hypothesized that the TdfJ loop three helix (L3H), which is identical in amino acid sequence to the ZnuD L3H (Figure 10), plays a similar role in binding and zinc extraction from S100A7.
Crystal structures of *N. meningitidis* ZnuD in multiple conformations according to metal ion presence. A) The apo form of the protein, which forms an α-helix finger in extracellular loop three. B) A cadmium-soaked ZnuD to simulate zinc presence. The helical region of loop three is rearranged into flexible β-strands upon metal ion binding, granting access to a high-affinity zinc binding site near the plug domain. C) Zinc-soaked ZnuD, showing zinc irons in the peripheral, high-affinity, and periplasmic binding sites. Structural gaps represent unresolved loop sections.

Adapted from (292).
Figure 9. The loop three helix is conserved in TbpA, where it aids iron piracy from transferrin

The structure of TbpA in complex with transferrin is shown in the background, with a zoomed-in box highlighting the loop three helix. TbpA is shown in green ribbon, the transferrin C-lobe in gold ribbon, the transferrin N-lobe in red ribbon, and the TbpA loop three helix in magenta ribbon. A red iron atom is shown in the cleft. During docking, the loop three helix inserts into the iron-binding cleft of the transferrin C-lobe, where it is thought to disrupt iron ligation.

Adapted from (252).
Figure 10. The ZnuD loop three helix shares its amino acid sequence with TdfJ

An amino acid sequence alignment from multiple *Neisseria* species shows the conservation of the two zinc-sensing clusters and the helical region of TdfJ/ZnuD loop three. Sequences from *N. meningitidis* (Nme) and *N. gonorrhoeae* (Ngo) are highlighted. Adapted from (292).
In this report, we determined the affinity of wild-type TdfJ and S100A7, then utilized site-directed mutagenesis to identify key amino acid residues of TdfJ involved in binding, and subsequent zinc extraction from S100A7.

II. Results

A. Wild-type TdfJ binds S100A7 with high affinity

We previously reported that whole gonococcal cells presenting TdfJ on their surface can bind S100A7 (249). However, the interaction was not investigated using purified proteins in isolation, suggesting that other membrane factors may play some role in binding. To address this question, we sought to further characterize the interaction between WT TdfJ and S100A7. To this end, we purified WT TdfJ and performed surface plasmon resonance (SPR) to interrogate binding of S100A7 to TdfJ (Figure 11A). His-tagged TdfJ was immobilized on a Ni-NTA sensor chip and subsequently blocked with His-tagged streptavidin. Successive injections of 1, 10, 50, 100, and 500 nM S100A7 were then utilized to characterize the binding interaction. Additions of S100A7 generated a concentration-dependent response ranging from approximately 220 average response units (RU) upon addition of 1 nM S100A7 to approximately 900 average RU for 500 nM S100A7. Analysis of these sensorgrams revealed a high-affinity interaction between TdfJ and S100A7. An EC50 calculation was used to generate a binding curve and the data were fit to a single-site binding model, yielding a dissociation constant (K\text{D}) of 41 nM (Figure 11B). These data corroborate our previous proposal that TdfJ interacts with S100A7 and indicate that the proteins themselves, devoid of any external membrane factors, bind with high affinity. With this in mind, we next sought to mutagenize the putative zinc-sensitive region of TdfJ and
Figure 11. Wildtype TdfJ binds S100A7 with high affinity
**Figure 11. Wildtype TdfJ binds S100A7 with high affinity.** His-tagged, wild-type TdfJ was immobilized on an NTA-coated SPR sensor chip, and the remaining free NTA sites were blocked with His-tagged streptavidin. S100A7 was then passed over the TdfJ chip to assess binding. (A) SPR sensorgram showing the baseline, association, and steady-state binding of five concentrations (1, 10, 50, 100, and 500 nM) of S100A7 with wild-type TdfJ. The binding detected is reported as arbitrary response units (RU) on the y axis. Gaps in the trace lines represent response spikes, likely generated by air bubbles, which were removed postrun. The plot is a representative trace for 3 experiments. (B) Plot showing the steady-state affinity of S100A7 and TdfJ calculated from multiple analyte injections of each concentration over multiple runs. A line of best fit is shown and was used to calculate a dissociation constant. Figure from (410).
assess its impact on S100A7 recognition. We first generated a deletion mutant lacking fifteen amino acids from the L3H (ΔL3H) to gauge whether the region in question was a suitable target. Then, with an understanding that such a mutant may have profound impacts on overall protein folding and stability, we focused on point mutations within the original fifteen residue segment. These mutations included proline substitution to physically disrupt the helical motif, and charge changes to potentially alter zinc coordination in either TdfJ or S100A7. A full list of L3H mutants is shown in Table 1.

B. Variant tdfJ genes were expressed from an inducible ectopic site in the gonococcal chromosome

The native locus of tdfJ is maximally expressed only during conditions of low zinc and high iron (249, 287). As such, reliably reproducing equivalent gene expression and protein production profiles for mutated tdfJ genes in the native locus promised to be a challenge that may confound comparisons between mutants. As such, we instead chose to perform experiments using the isogenic tdfJ mutant strain MCV928 (402), which has an inactivated native locus, and to add back our mutated tdfJ genes via the complementation plasmid pVCU234, which contains a strong ribosome binding site behind a promoter inducible by isopropyl β-d-thiogalactopyranoside (IPTG) (411). A schematic of the final genotype is shown in Figure 12A, and unless otherwise noted, a “WT” strain in this report refers to an unmutated tdfJ expressed from the complementation locus, not a true WT strain. This method hypothetically allowed more precise control of protein production via addition of a consistent amount of inducer, and therefore more consistent comparisons of protein characteristics.
**Table 1. Mutagenesis of TdfJ Loop 3 Helix Residues**

<table>
<thead>
<tr>
<th>Position</th>
<th>Wild Type</th>
<th>Mutation(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full Helix</td>
<td>(^{255}\text{QKSLINKRYLQLYPHL}^{270})</td>
<td>Deletion</td>
</tr>
<tr>
<td>255</td>
<td>Glutamine</td>
<td>Proline</td>
</tr>
<tr>
<td>256</td>
<td>Lysine</td>
<td>Glutamate</td>
</tr>
<tr>
<td>261</td>
<td>Lysine</td>
<td>Glutamate, Proline</td>
</tr>
<tr>
<td>262</td>
<td>Arginine</td>
<td>Proline</td>
</tr>
<tr>
<td>263</td>
<td>Tyrosine</td>
<td>Proline</td>
</tr>
<tr>
<td>265</td>
<td>Glutamine</td>
<td>Proline</td>
</tr>
<tr>
<td>266</td>
<td>Leucine</td>
<td>Proline</td>
</tr>
<tr>
<td>269</td>
<td>Histidine</td>
<td>Aspartate, Proline</td>
</tr>
<tr>
<td>261/262</td>
<td>Lysine/Arginine</td>
<td>Proline/Proline</td>
</tr>
<tr>
<td>262/263</td>
<td>Arginine/Tyrosine</td>
<td>Proline/Proline</td>
</tr>
<tr>
<td>265/266</td>
<td>Glutamine/Leucine</td>
<td>Proline/Proline</td>
</tr>
</tbody>
</table>
Figure 12. Variant *tdfJ* genes are expressed from an inducible ectopic site in the gonococcal chromosome.
Figure 12. Variant *tdfJ* genes are expressed from an inducible ectopic site in the gonococcal chromosome. Wild-type and mutated forms of *tdfJ* were cloned into a complementation vector designed to insert into an ectopic site of the gonococcal chromosome between the *aspC* and *lctP* loci. This construct contains an IPTG-inducible promoter and a strong ribosome-binding site upstream of the inserted gene. (A) Schematic demonstrating the final genotype of gonococcal *tdfJ* mutants. Plasmids containing either wild-type or mutated *tdfJ* were used to transform N. gonorrhoeae strain FA19 with its native *tdfJ* locus inactivated by an omega cassette, resulting in gonococci that produce only TdfJ under induction. (B) Gonococcal *tdfJ* mutants grown on GCB agar plates with (+) and without (−) 1 mM IPTG were resuspended in PBS. Cell suspensions were standardized and used to prepare cell lysates, which were Western blotted (WB) to assess TdfJ production and the IPTG control. A true wild-type strain, FA19, and its isogenic *tdfJ* mutant were grown under zinc-limited conditions to serve as positive and negative controls. Ponceau staining (P.S.) of the blots is also shown to demonstrate equal loading. (“FA19 WT” refers to the true wild-type strain expressing *tdfJ* from its native locus; “WT” refers to the wild-type *tdfJ* gene in the inducible ectopic site; and “ΔL3H” refers to a deletion mutant in *tdfJ*, which lacks 15 amino acids from the loop 3 α-helix.) The blot is representative of results from 3 experiments. Figure from (410).
After generating the inducible mutants, we verified that they produced the correct gene product under IPTG control, as predicted, by performing western blot analysis of gonococcal \textit{tdfJ} mutants grown with and without IPTG to detect TdfJ production (Figure 12B). A true WT strain FA19 (401) was also grown in low zinc, high iron conditions to serve as a positive control, and a lysate of strain MCV928 was used as a negative. The blots showed that each mutant strain overexpressed its respective \textit{tdfJ} gene only when IPTG was present, and no protein was detected when inducer was absent. With these conditions established, we moved on to further characterization of the mutants.

\textbf{C. TdfJ mutants are surface exposed and mimic the folding of the wildtype}

Before performing S100A7 binding assays, we ensured the mutations we introduced into \textit{tdfJ} did not have profound, off-target impacts on TdfJ stability or its presentation on the cell surface, as these would have confounded our analyses. To assess this, we first characterized a panel of TdfJ-specific mouse monoclonal antibodies (mAbs) and identified those that recognized either folded, surface-exposed TdfJ, western blotted TdfJ, or both (Figure 13). These validation experiments indicated that only gonococci producing TdfJ, either as whole cells or as lysates, were recognized by the mAbs, as the \textit{tdfJ} knockout strain was not detected above background levels. The capacity for recognizing TdfJ on the gonococcal cell surface made the mAbs a useful tool for assessing the global fold of mutated forms of TdfJ. We immobilized gonococci producing these mutated proteins on a nitrocellulose membrane and probed their surface with mAbs to compare to the pattern for the WT (Figure 14). We also probed a strain not producing TdfJ to demonstrate specificity. All strains that produced a form of TdfJ were recognized, suggesting they successfully
Figure 13. Characterization of TdfJ-specific mouse monoclonal antibodies

(A) Gonococcal WT and tdfJ knockout (tdfJ KO) strains were grown on GCB agar plates containing 1 mM IPTG and then resuspended in PBS at an OD$_{600}$ of 1.0. Cell suspensions were dotted onto nitrocellulose in a dot blotter and allowed to adsorb and dry. The dot blot was blocked with BSA and subsequently probed with either HRP-conjugated S100A7 (S100A7-HRP) or TdfJ-specific mouse monoclonal antibodies (mAbs) in a series of dilutions (0.4 → 0.2 → 0.1 → 0.05 μM for S100A7-HRP and 1:10 → 1:50 → 1:100 for mAbs). The blots were then probed with either HRP-conjugated anti-mouse IgG secondary antibodies, and the signal was developed by the addition of the HRP-reactive CN/DAB substrate (for mAbs 4-2E2, 4-2A2, 4-3F11, and 4-5E11), or AP-conjugated anti-mouse IgG secondary antibodies, and the signal was developed by the addition of the AP-reactive NBT-BCIP substrate (for 1-2B11 and 1-8H4). (B) WT and tdfJ KO strains were grown as described above, and whole-cell lysates were prepared for SDS-PAGE and Western analysis. Western blots were probed with either anti-TdfJ polyclonal serum (α-TdfJ), one of the six mAbs, or all antibodies pooled for the tdfJ KO lysate. Ponceau staining (P.S.) of the blots is shown to demonstrate equal sample loading. Adapted from (410).
Figure 14. TdfJ mutants are surface exposed and mimic the folding of the wild type

Gonococcal mutants were resuspended from solid medium supplemented with IPTG and dotted onto nitrocellulose in standardized amounts. After blocking, cultures were probed with the six monoclonal antibodies described in Figure 4.6 and detected using HRP-labeled secondaries.

Blot is representative of N=3 experiments. Adapted from (410).
exported the protein to the surface and that mutation did not affect the TdfJ interaction with the Sec system or the β-barrel assembly machinery (reviewed in (412)). The mAb binding pattern for WT TdfJ remained consistent across mutant strains and was indistinguishable from the WT for most mutants, suggesting the overall fold and stability of the protein was not perturbed by mutagenesis.

D. Mutations in the TdfJ loop 3 α-helix can inhibit S100A7 binding

To assess TdfJ mutants for their S100A7 binding phenotypes, we first performed a broad screen using whole cells expressing TdfJ. As before, induced gonococcal cultures were dotted to nitrocellulose, and were then probed with a dilution series of HRP-labeled S100A7 (S100A7-HRP) (Figure 15). We found that certain mutations diminished S100A7 binding to varying degrees. The most profound defects were seen in the ΔL3H strain and in a mutant with residues K261 and R262 replaced with prolines (K261P/R262P), both of which generated no detectable S100A7 binding. Minor to moderate binding impacts were also seen in the K256E, R262P, Q265P, L266P, and Q265P/L266P mutants. To investigate these defects further, we purified the above listed TdfJ proteins and utilized an ELISA-based binding assay to assess their defects. A protein-stained SDS gel showing the purity of recombinant TdfJ is shown in Figure 16. His-tagged versions of these proteins were purified and immobilized in a Ni-NTA-coated 96-well plate and were subsequently probed with S100A7-HRP (Figure 17A). Consistent with the dot blot results, the ΔL3H and K261P/R262P mutants exhibited virtually no S100A7 binding; their $A_{450}$ readings were indistinguishable from samples with no probe or no target protein added. Additionally, the R262P, Q265P, and L266P mutants demonstrated results consistent with the dot blot analyses as well, and
Figure 15. Mutations in the TdfJ loop 3 α-helix can inhibit S100A7 binding

Gonococcal *tdfJ* mutants were grown on GCB agar plates containing 1 mM IPTG and resuspended in PBS to an OD$_{600}$ of 1.0. Suspensions were dotted onto nitrocellulose and allowed to adsorb and dry prior to blocking. The blot was then probed with a dilution series of HRP-labeled S100A7, and the signal was developed with the HRP-reactive CN/DAB substrate. Mutants with appreciable decreases in S100A7 binding are color-coded, and the color convention is maintained for the remainder of chapter 4. The blot is representative of results from 3 experiments. Adapted from (410).
**Figure 16. Purification of recombinant TdfJ**

A protein-stained gel showing the purification of recombinant TdfJ. The far-left lane shows protein molecular weight markers, followed by lanes depicting (from left to right) column flowthrough and a wash/elution series ranging from 0 to 300 mM imidazole. TdfJ eluted cleanly at 300 mM imidazole, and appears as a single band at ~85 kDa.
Figure 17. Purified TdfJ forms retain the structure and binding characteristics of the membrane-bound forms
Figure 17. Purified TdfJ forms retain the structure and binding characteristics of the membrane-bound forms. (A) Mutant forms of TdfJ determined to have binding deficiencies based on the results from Figure 4.8 were purified and used in a quantitative binding assay with S100A7. Purified, His-tagged TdfJ variants were seeded onto an NTA-coated 96-well plate and then blocked with BSA. They were then probed with S100A7-HRP and washed, and the signal was developed with the TMB substrate. The signal was quantified by reading the absorbance at 450 nm, and all samples were calculated as a percentage of the WT. Statistical differences between mutants and the WT were calculated via one-way analysis of variance (ANOVA) (ns, not significant; *, P < 0.05; **, P < 0.01; ****, P < 0.0001). (B) After purification, mutant forms of TdfJ were assessed for stability and folding. Proteins were seeded into a Ni-NTA-coated 96-well plate and subsequently probed with the mAbs described in the legend of Figure 4.6. mAb binding was detected via the addition of HRP-labeled anti-mouse IgG, and the signal was quantified by reading the A450. His-tagged TdfH was added as a control for antibody specificity, and a set of proteins was probed with no primary antibody to assess the background. Statistical differences between the WT and mutants were assessed via two-way ANOVA, and all mutants were statistically indistinguishable from the WT for each mAb. Means and standard deviations from 3 experiments are shown for panels A and B. Adapted from (410).
bound S100A7 at levels that were statistically different from WT, but not as profoundly impacted as the previous two. The Q265P/L266P double mutant was somewhat inconsistent between experiments; this mutant appeared roughly similar to the R262P, Q265P, and L266P single mutants in the dot blot, but performed significantly worse in the ELISA assay. The K256E mutant was not statistically distinguishable from WT.

To ensure that the binding defects seen in the ELISA assay were not due to protein misfolding or instability induced during purification, we utilized the TdfJ-specific mAbs to probe purified TdfJ mutant proteins. The His-tagged TdfJ mutants were seeded into a Ni-NTA-coated ELISA plate and subsequently probed with the same set of mAbs that were used in the surface-exposure test (Figure 17B). Notably, all TdfJ variants were recognized by each of the mAbs at levels that were statistically indistinguishable from that of the WT, suggesting that the global fold of the purified proteins mimicked that of their membrane-bound counterparts, and that the mutated forms were as well folded as the WT protein. His-tagged TdfH was used as a negative control to demonstrate mAb specificity for TdfJ and was not recognized by the mAbs. The results of these experiments suggest that any differences observed in binding interactions with S100A7 for the mutated forms of TdfJ were not likely to be structural in nature.

We next focused on the mutants that were most defective in our previous binding assays, namely ΔL3H and K261P/R262P. While both mutants showed similar binding deficiency, we chose to utilize only K261P/R262P for affinity calculations, as the point mutant was a better representative overall for TdfJ as opposed to ΔL3H, which contained a large deletion. We performed SPR as for the WT protein, using His-tagged K261P/R262P
TdfJ as the sensor chip ligand and a 1-500 nM analyte series of S100A7 (Figure 18A). These experiments confirmed the results of our earlier assays, namely that K261P/R262P was severely defective in S100A7 binding capability. At the highest concentration of analyte added (500 nM), K261P/R262P generated approximately 325 average RU at equilibrium, only marginally more than was seen for 1 nM S100A7 on WT TdfJ, and substantially less than was seen for the same analyte concentration on WT (~900). K261P/R262P generated a very slight concentration-dependent response when S100A7 was added, suggesting that its binding signal, while small, was specific to S100A7 and not merely an artifact of analyte injection in general. As with the WT, we attempted an affinity calculation for K261P/R262P (Figure 18B). However, fitting of the data to the single-site model as used to extract the affinity for the WT protein failed to provide a reasonable fit, and no reliable K_D calculation could be made. While the data could likely fit to a more complex model with more variables, the inability to fit the data to the simpler model was attributed to much lower sensitivity of the data as a result of the very small range of the response in the sensorgrams and we concluded an alternate model was not merited.

E. Gonococci expressing mutated tdfJ are defective for S100A7 utilization

As a consequence of their impaired binding, we next investigated whether other known TdfJ/S100A7 interaction phenotypes were also impacted by mutagenesis. We previously reported that gonococci producing a functional TdfJ are able to utilize Zn-loaded S100A7 (Zn-S100A7) as a sole zinc source in metal-depleted medium, and that growth in the presence of Zn-S100A7 leads to zinc accumulation within gonococci in a TdfJ-dependent way (249). To determine whether binding deficiencies affected downstream utilization of
Figure 18. The K261P/R262P TdfJ mutant has a severe S100A7 binding deficiency

His-tagged K261P/R262P TdfJ was immobilized on an NTA-coated SPR sensor chip, and the remaining free NTA sites were blocked with His-tagged streptavidin. S100A7 was then passed over the TdfJ chip to assess binding. (A) An SPR sensorgram showing the baseline, association, and steady-state binding of five concentrations (1, 10, 50, 100, and 500 nM) of S100A7 with K261P/R262P TdfJ. The plot is a representative trace for 2 experiments. (B) Plot showing the steady-state affinity of S100A7 and K261P/R262P TdfJ calculated from two individual traces for each analyte concentration. Affinity could not be calculated using the same model as the one used for the WT in Figure 11. Figure from (410).
Zn-S100A7, we performed growth assays in the same metal-restricted medium as before, supplemented with Zn-S100A7, and compared growth of mutant strains to that of the WT (Figure 19). In this assay, all strains grew equivalently when supplemented with ZnSO₄ instead of Zn-S100A7, suggesting that baseline growth defects were not present in the TdfJ mutants, and that zinc utilization in general was not compromised when a suitable zinc source was present. When samples were fully restricted for zinc by addition of the zinc-specific chelator TPEN no growth was observed for any strain. However, when the TPEN-treated samples were also supplemented with Zn-S100A7, gonococci producing TdfJ variants (+ IPTG) exhibited varying capacity for growth, consistent with their binding phenotypes. Unsurprisingly, WT TdfJ facilitated the most growth, while the ΔL3H and K261P/R262P samples reached similar optical densities to their TPEN-only counterparts, suggesting effectively no capacity for Zn-S100A7 use. Likewise, R262P, Q265P, L266P, and Q265P/L266P fell between the two extremes, consistent with our findings in the binding assays.

Finally, we assessed whether TdfJ mutation affected zinc accumulation within the gonococci when Zn-S100A7 was present. We grew cultures in zinc-restricted medium supplemented with Zn-S100A7 and harvested cell pellets. After removing exogenous metals, cell pellets were analyzed for their zinc content by inductively-coupled plasma mass spectrometry (Figure 20). These experiments were consistent with all others and showed that WT TdfJ facilitated the most zinc uptake, while ΔL3H and K261P/R262P allowed the least. All other mutants fell between the extremes as they had done in other assays. Taken together, these data suggest that the α-helical region of TdfJ loop three, situated between
Figure 19. Gonococci expressing mutated 
*tdfJ* are defective for S100A7 utilization

Gonococcal strains were grown in metal-restricted defined medium until they reached exponential phase to induce zinc starvation. Cells were then back-diluted to an OD$_{600}$ of 0.02 in the same medium and added to 96-well plates supplemented with either ZnSO$_4$, TPEN, or TPEN plus zinc-loaded S100A7 with and without IPTG. Cells were grown for 12 h, with the OD$_{600}$ being recorded every 30 min to assess growth. Statistical significance relative to the WT was calculated via two-way repeated-measures ANOVA with Geisser-Greenhouse correction (ns, not significant; **, $P < 0.01$; ***, $P < 0.001$). Means and standard deviations from 3 experiments are shown. Figure from (410).
Gonococcal *tdfJ* mutants were grown in metal-restricted medium until exponential phase to induce zinc stress. Cultures were back-diluted in the same medium and supplemented with TPEN and zinc-loaded S100A7, with IPTG added where appropriate. Cultures were grown for an additional 4 h, and cell pellets were then collected via centrifugation. Pellets were washed twice with buffer containing 10 mM HEPES plus 1 mM EDTA and then once more with 10 mM HEPES only. The cell pellets were digested, and the zinc content was assessed via ICP-MS.

Statistical differences relative to the WT with IPTG were calculated via one-way ANOVA (*, $P < 0.05$; **, $P < 0.01$). Means and standard deviations are shown for 6 experiments for the two WT samples and 3 experiments for all others. Figure from (410).
two His-, Asp- and Glu-rich clusters, plays an essential role in binding of, and subsequent zinc extraction from, S100A7.

III. Discussion

Gonorrhea presents a serious threat to public health, as there is currently no licensed vaccine against the disease and highly-drug-resistant isolates of the causative agent, *N. gonorrhoeae*, continue to emerge. During this pathogen’s lifecycle within the human host, it is confronted by host efforts to limit availability of critical nutrients such as iron and zinc – a concept termed “nutritional immunity” – in order to starve out the infection. In response, the gonococcus deploys eight TdTs to its outer-membrane, which serve the critical function of overcoming host nutritional immunity efforts by binding host factors and pirating their metal cargo (reviewed in (413)). The TbpAB and LbpAB systems facilitate iron acquisition from human transferrin and lactoferrin, respectively (267, 268, 414); the HpuAB system allows the gonococcus to extract iron from hemoglobin and hemoglobin-haptoglobin complexes (274, 275); FetA scavenges xenosiderophores from other bacterial species and co-opts them for gonococcal use (281); TdfH facilitates zinc acquisition from the innate immunity protein calprotectin (287); two other TdTs, TdfF and TdfG, do not have a known ligand, but both are repressed by iron, suggesting a role in iron uptake (295). Finally, TdfJ, the topic of this report, is responsible for gonococcal zinc piracy from S100A7 (249).

In this study, we quantified the binding interaction between TdfJ and S100A7 and found a high affinity, with a $K_D$ of 41 nM. This is consistent with other interactions between gonococcal TdTs and their host ligands. For example, TdfH binds calprotectin with
nanomolar affinity (288), and a gonococcal strain producing only TbpA and no TbpB binds transferrin with a $K_d$ of approximately 10 nM (256). Additionally, the lipoprotein component of the lactoferrin-iron uptake system, LbpB, binds lactoferrin with a $K_d$ of 140 nM (270).

Such high-affinity interactions are not surprising, as more efficient uptake of iron and zinc during infection would hypothetically offer an evolutionary advantage to the gonococcus. However, it is noteworthy that such strength of interaction may not be entirely necessary, as gonococci seem likely to encounter an abundance of calprotectin and S100A7 during infection. *N. gonorrhoeae* characteristically stimulates a local influx of neutrophils during infection (388, 390), and calprotectin is abundant within the neutrophil cytosol (243). Similarly, S100A7 is upregulated in response to inflammation, and is known to be present in the female genital tract (241), suggesting that it too should co-localize with invading gonococci.

As mentioned, the crystal structure of meningococcal ZnuD served as the roadmap for our mutagenesis studies, and we focused our efforts on an $\alpha$-helix motif situated between two zinc-sensing clusters in extracellular loop three. As discussed (292), this region undergoes considerable remodeling in a substrate-dependent way, with both zinc and cadmium presence causing the exposed $\alpha$-helix to collapse into $\beta$-strands, presenting a buried, high-affinity zinc site. Because of the implied importance of this region to zinc uptake, we hypothesized that it may be involved in binding to and/or piracy from S100A7. We primarily pursued two routes for mutagenesis. First, we generated a fifteen-residue deletion in loop 3 between the zinc-sensitive clusters where the $\alpha$-helix is found. This mutation effectively served as proof of concept for targeting this region, as any defects
should have been apparent from such a significant change. Second, because of the unique architecture of ZnuD as concerns its loop remodeling capabilities and subsequent zinc import, we sought mutations that would cause disruption to this specific TdfJ motif with minimal impact on the rest of the protein. To this end, we elected proline substitution, as this cyclic amino acid has high conformational rigidity and is known to disrupt helical secondary structure (415). Mutations of a few charged residues were also designed, with the goal of disrupting electrostatic contributions to the binding of S100A7 and/or perturb metal coordination in one or both proteins. Interestingly, mutations within the α-helix generated variable levels of binding deficiency, with severe defects seen upon proline insertion at residues K261 and R262, which are located centrally within the helix.

The importance of an α-helix in a TdT extracellular loop is not unique to TdfJ. The crystal structure of meningococcal TbpA in complex with transferrin shows such a helix finger in close proximity to the transferrin C-lobe, where it is thought to disrupt iron coordination (252). As mentioned, Cash et al. (254) mutagenized this region and found that certain mutations diminished TbpA/transferrin interactions in the gonococcus, though proline insertion was not used. A recent report also posited the importance of the TbpA helix in transferrin binding. Duran et al. (253) performed molecular dynamics simulations to interrogate the docking of TbpA and transferrin, and found that the TbpA loop three helix undergoes structural rearrangement upon transferrin binding. The authors hypothesized that this dynamic allows K359 of TbpA to interact with D634 of transferrin, ultimately resulting in a charge repulsion between transferrin residues K534 and R632, thus opening the binding cleft to free the iron atom. Such a mechanism may be possible for TdfJ and
S100A7 as well, though it has not yet been tested. It is important to note that the respective helices of these TdTTs do not share appreciable sequence similarity (TbpA: \(K^{351}\text{AVFDANKQA}^{361}\); TdfJ: \(Q^{255}\text{KSLINKRYLQLYPH}^{269}\)), suggesting that the helical structure itself is the predominant conserved property, but further investigation of other TdT/ligand pairs would be needed to validate this.

The TdTTs have received considerable interest as vaccine targets for gonorrhea, and TdfJ/ZnuD is no exception to this (291). The TdTTs are well-conserved across gonococcal isolates and show limited propensity for antigenic variation, which has stymied efforts targeting many other surface structures. In addition to their conserved nature, the importance of the TdTTs to gonococcal infection cannot be overlooked. A \(N.\ gonorrhoeae\) strain FA1090 with an inactivated transferrin receptor system is unable to cause infection in human males (266); TdfH allows gonococci to survive within neutrophil NETs (287); TdfF is required for gonococci to replicate within cervical epithelial cells (297); ZnuD contributes to meningococcal interactions with epithelial cells, and \(znuD\) mutants are defective for dissemination in a mouse model (292, 294). Such key virulence factors are highly promising as vaccine and/or therapeutic targets, and mutagenesis of such targets has received attention with regards to generating a robust, protective immune response. A few recent studies have focused on identifying mutated forms of bacterial structures that are unable to bind the host ligands that are recognized by their wild-type counterparts, acting on the hypothesis that a bound host factor may dampen the immune recognition of said bacterial target. Such studies by Frandoloso et al. and Martínez-Martínez et al. (335, 416) mutagenized the TbpB protein found in the pig pathogen \(Haemophilus parasius\), and
demonstrated that a transferrin-binding-defective mutant, which retained a wild-type-like conformation, conferred superior protection against bacterial challenge compared to WT TbpB, and also elicited a more robust B- and T-cell response. Beernink et al. similarly generated non-binding mutants of meningococcal factor H binding protein (fHbp) and found upon immunizing factor H transgenic mice that a nonbinding mutant elicited more bactericidal antibodies and more factor H blocking antibodies than was seen for WT fHbp (385). However, the mutagenesis paradigm does not appear to be universally true, as earlier fHbp mutagenesis efforts by Beernink et al. showed an impaired immune response and compromised a key immunogenic region of fHbp (417). To date, no studies have been published which investigate whether a nonbinding version of a TdT may confer superior protection and/or immune stimulation, so one must be cautious about discussing their potential superiority as antigens. Nevertheless, a future study utilizing such TdT mutants, including the one(s) described in this report, may be an interesting path moving forward.

In summary, we herein report for the first time the binding interaction for gonococcal TdfJ and its human ligand S100A7. We also identified several mutations in TdfJ loop three that alter S100A7 binding and subsequent zinc extraction. Because the TdTs continue to feature as promising targets for vaccine and therapeutic development to combat gonococcal disease, an intimate understanding of their virulence mechanisms is of paramount importance, and similar characterization of the other TdT/ligand pairs will hopefully follow.
CHAPTER 5: CHARACTERIZATION OF TDFJ AS A GONOCOCCAL VACCINE ANTIGEN

I. Introduction

As mentioned in previous sections, the TdTJs have received considerable attention as vaccine targets to prevent Neisserial disease. The proteins are well conserved, play critical roles in infection and bacterial survival, and are surface-accessible. To date, the vaccine potential of the TbpAB complex is the most thoroughly studied. For example, in the meningococcus these proteins have demonstrated capacity to protect against infection and elicit a protective antibody response in laboratory animals (418, 419), and TbpB especially garnered considerable attention as the more immunogenic of the two partners (420, 421). Concordant with the observations about meningococcal TbpB immunogenicity, experiments with the gonococcus showed a similar result. In one study, full-length TbpA and TbpB were coupled to the B subunit of cholera toxin and utilized in intranasal mouse immunization (422). These experiments demonstrated that again, anti-TbpB titers were higher, but those against TbpA were more cross-reactive, likely due to the protein’s higher levels of conservation. Promisingly, these conjugates were shown to induce both serum and vaginal antibodies, with bactericidal activity observed in serum antibodies, suggesting their utility as vaccine antigens was worthwhile to pursue. In a follow-up study, specific regions of TbpA and TbpB were utilized, with the TbpB [transferrin-binding] N-lobe and extracellular loop 2 of TbpA grafted onto the A2 subunit of cholera toxin, thus creating a chimeric hybrid. These preparations similarly induced an antibody response with bactericidal activity, and also inhibited transferrin-dependent growth when co-incubated with iron-stressed gonococci.
(337). This indicated that, beyond whole proteins, individual loops of TbpA could induce functional anti-gonococcal antibodies that targeted whole TbpA.

Such a platform was enticing for a multitude of reasons. First, a large (industrial)-scale vaccination effort promises to require considerably large quantities of intact, high-quality antigen. Integral membrane proteins, however, are expressed at low levels in bacterial membranes and require delicate detergent conditions for stabilization and solubilization, which may bottleneck production. Certain lipoproteins, however, possess a useable β-barrel which can feasibly act as a surrogate host for extracellular TdT loops, and are reliably produced in large quantities in bacterial cytoplasm with no specific solubilization needs. Second, a highly-cross-reactive antigen can ostensibly be engineered by selecting highly conserved TdT loop sections for inclusion in the hybrid, enhancing the efficacy of vaccination against heterologous strains. In a recent study, this concept was investigated thoroughly via the construction of multiple TbpB/TbpA loop hybrids. Fegan et al. utilized the C-terminal domain of TbpB as a scaffold to host TbpA loops, and found that antisera from immunized animals recognized whole TbpA expressed by *N. meningitidis*, and observed antibody functions including blocking transferrin-dependent growth and bactericidal activity (336). Furthermore, a hybrid hosting TbpA loop 10 was as effective as whole recombinant TbpA at limiting gonococcal colonization in the mouse lower genital tract. Beyond the *Neisseria*, this lipoprotein/TdT loop hybrid platform has shown success in vaccination against other pathogens as well. Hybrid antigens using the aforementioned TbpB C-lobe with extracellular loop sections from the *Acinetobacter baumannii* TdT ZnuD were recently evaluated in a sepsis model to assess protection. Hybrids expressing
individual loops showed between 25 and 50% protection, while a four-loop hybrid was 100% effective at preventing *Acinetobacter* infection (423).

Beyond merely antigen selection, however, choice of adjuvant can have critical implications on overall vaccine efficacy. The studies referenced above followed similar paths regarding antigen selection and/or construction, but choice of adjuvant was unique for each. (Price et al., cholera toxin A or B; Fegan et al., alum; Qamsari et al., CFA). While to date no prototypical immune state to protect against gonococcal infection has been established, recent work demonstrated that cytokine-mediated modulation of the immune response can induce protection against *N. gonorrhoeae* (357, 358, 391). The experiments in this chapter aimed to assess two questions: 1) Is the lipoprotein-based hybrid antigen platform a suitable method to host TdfJ loops, and if so, what are the results of immunizing with such an antigen? 2) Does whole recombinant TdfJ formulated with different adjuvants elicit differential responses with respect to protection and antibody generation? To that end, we generated a series of hybrid antigens hosting TdfJ loop sections on a *tbpB* ortholog from *Vibrio cholerae* (VcSLP) and utilized them for a series of mouse immunizations, after which antisera and vaginal lavages were screened for functional antibodies. In addition, we formulated recombinant TdfJ with four adjuvants prior to mouse immunization and assessed their capacity to protect mice in a genital challenge model.

II. Results

A. Loop Selection and Hybrid Antigen Design

We first set out to define the loop sections of TdfJ that would be used for insertion onto the VcSLP scaffold. To this end, we utilized the crystal structure of meningococcal
ZnuD (292) to isolate known extracellular loop sections, as no high-resolution structure with confirmed topology for TdfJ is currently available. Once loop sequences were confirmed, we conducted a variety of bioinformatic predictions to identify potential B-cell epitopes within these sequences. Our predictions utilized the following servers: ABCpred (424), CBTOP (425), Ellipro (426), SVMTriP (427), IEDB (428), LBTope (429), DiscoTope 2.0 (430), and BepiPred 2.0 (431). These results were aligned and sections with highest incidence of prediction were selected. In total, peptides from all extracellular loops with the exception of loop one were selected, and their sequences can be found in Table 2.

The e5460 plasmid contains a modified version of the VcSLP gene with four long loop sections removed and replaced with short linker peptides, designated sites 1-4. For hybrid engineering, we utilized the BioXP system (Codex DNA) to synthesize both gene segments for the TdfJ peptides and VcSLP gene fragments up and downstream of the insertion sites, followed by five DNA assembly reactions to generate five hybrid genes with TdfJ loops in VcSLP sites 1, 2, 3, and 4. A schematic image of the overall workflow is shown in Figure 21. Following fragment assembly, hybrid genes were inserted back into an empty e5460 backbone via complementary end joining ligation and used to transform *E. coli*. Correct gene insertion was verified by PCR.

Antigen design and synthesis was a combined effort between myself and Somshukla Chaudhuri, and occurred in the laboratory of Dr. Anthony Schryvers at the University of Calgary.
Table 2. TdfJ Hybrid Loop Peptides

<table>
<thead>
<tr>
<th>TdfJ Loop</th>
<th>Peptide</th>
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<tbody>
<tr>
<td>2</td>
<td>GDYVRGRLKNLPSLPGREDAYGNRFIAQDDQNAVRVPARL</td>
</tr>
<tr>
<td>3</td>
<td>DRRDQYGLPAHSHEYDDCHADIIWQKSLINKRYQLYPHLTEEDIDYNP GLSCGFHDDDNAHAHTSHGRPWIDLRNK</td>
</tr>
<tr>
<td>4</td>
<td>DYRHDEKAGDAVENFFNNQ</td>
</tr>
<tr>
<td>5</td>
<td>QQKSSALSAISEAVKQPMDDNQKVQ</td>
</tr>
<tr>
<td>6</td>
<td>VEKQKASIQYDKNALRENNYNYHNLPLGDIARRQTA</td>
</tr>
<tr>
<td>7</td>
<td>ERLPSTQELYAHGHVATNTFEVGNKHLNKER</td>
</tr>
<tr>
<td>8</td>
<td>RFGNYYIAYTLDNDRGPKSIDDESMKLRVYGNQSGAD</td>
</tr>
<tr>
<td>9</td>
<td>GDYVRGRLKNLPSLPGREDAYGNRFIAQDDQNAVRVPARL</td>
</tr>
<tr>
<td>10</td>
<td>RVFAQNKLARYETTGH</td>
</tr>
<tr>
<td>11</td>
<td>ADNLLNQSVYAHSSFLSDTQPMGRSF</td>
</tr>
</tbody>
</table>
The amino acid sequence of TdfJ was submitted to online prediction software to detect putative B-cell epitopes in the extracellular regions. The nucleotide sequences of these epitopes were identified and synthesized \textit{de novo}, then submitted into a Gibson assembly experiment to graft them into the insertion sites of a lipoprotein scaffold (VcSLP). Assembled plasmids were used to transform \textit{E. coli}, and clones were screened for \textit{tdfJ} hybrid genes by PCR. Adapted from (292) and (336).
B. Antigen Purification

For hybrid antigen purification, the hybrid genes were amplified from their native plasmid and inserted into pET-19b, which encodes an N-terminal His-tag cleavable by enterokinase, and transferred to C41 (DE3) *E. coli*. After clones were recovered and confirmed by sequencing, we confirmed production of the His-tagged antigen by growing cultures in liquid medium with and without induction by IPTG. We then harvested whole-cell lysates which were assessed for presence of the His-tagged proteins at the predicted size via western blot (Figure 22). Once expression was confirmed, we attempted to purify the hybrid antigens. To this end, expression cultures were grown with inducer for approximately 4 h before cell pellets were harvested, lysed, and incubated with Ni-NTA resin for affinity capture. However, multiple iterations of the purification in different conditions (with and without inducer, Ni-NTA or Co-NTA resin, various buffers, etc.) yielded no soluble protein identifiable in elutions from the resin. A representative protein-stained gel is shown in Figure 23. It is hypothesized that the loop sections utilized in the antigen design, when fused into all four VcSLP insertion sites, generated proteins that were unstable and/or insoluble, and therefore either degraded or did not accumulate in detectable amounts in the *E. coli* cytoplasm. To circumvent this, we elected to isolate and solubilize *E. coli* inclusion bodies, which were thought to be the likely location of the misfolded proteins. After expression cultures were lysed, we collected any remaining insoluble material and isolated inclusion bodies, which were subjected to chaotropic solubilization. The dissolved inclusion bodies were assessed for their purity and predicted size on a Ponceau-stained membrane, which is shown in Figure 24. Note that no attempt was made to re-fold these
DE3 *E. coli* strains housing pET-19b-based expression plasmids for the TdfJ hybrids were grown in Terrific Broth until their OD$_{600}$ reached ~0.8 – 1.0, and were then induced with 1 mM IPTG.

Cultures were grown for 4 h, the cell pellets were harvested for lysates, and lysates were submitted to SDS-PAGE and western blotting using anti-his-tag antibodies to assess production of the hybrid antigens. Uninduced (- IPTG) lysates are also shown.
Figure 23. TdfJ hybrids did not purify in soluble form

A protein-stained SDS gel showing no pure fractions of the TdfJ 2,5,7,11 hybrid. Cultures expressing the TdfJ 2,5,7,11 hybrid were lysed and incubated with Ni-NTA, which was collected in a chromatography column, washed, and eluted with a gradient of 50 mM – 1M imidazole. The far left lane shows protein molecular weight markers, followed by the column flowthrough and elutions. No bands are seen in any elution.
Figure 24. TdfJ hybrids were isolated as solubilized inclusion bodies

A Ponceau-stained nitrocellulose membrane shows isolated, solubilized inclusion bodies for the five TdfJ hybrids. Expression cultures were harvested and lyse, and the remaining insoluble pellet was collected. This pellet was washed with PBS pH 8.0 + 1% Triton X-100 and 5 mM EDTA, then dissolved with 8M urea, 0.5% sarkosyl, and 1 mM DTT, then dialyzed against 2000-fold excess PBS pH 8.0. The membrane shows a cell lysate (left) and a sample of solubilized inclusion body (right) for each hybrid.
proteins, as no assay exists to assess the folding of these chimeras; they do not exist in nature and have no known detectable functions. While unfolded antigens are not ideal, we hypothesized that they would be digested and presented on MHC molecules as short peptides regardless, and therefore “correct” conformation may not be explicitly necessary. Whole recombinant TdfJ was produced by C41 (DE3) *E. coli* without the presence of inducer and was successfully purified by affinity chromatography from a Ni-NTA column as described in the methods. Collectively, these six antigens were utilized in mouse immunizations.

**C. Mouse Immunizations**

All mouse immunizations were performed at the University of Toronto in the laboratory of Dr. Scott Gray-Owen, with input from the laboratory of Dr. Trevor Moraes. Vaccine formulation, immunizations, experimental infections, CFU enumeration, and collection of serum and lavage samples were performed by Drs. Jamie Fegan, Elissa Currie, and Joseph Zeppa. Formulated vaccines were subjected to biophysical and biochemical analysis by Drs. Gregory Cole, Epshita Islam, and Dixon Ng to ensure stability and correct folding of TdfJ after admixing.

The solubilized VcSLP hybrids were formulated with alum in 25 μg doses. These formulations were administered intraperitoneally (IP) to female C57Bl/6 WT mice in three-week intervals, with four mice used per group. Groups included the five hybrids, VcSLP only, and alum only. Prior to immunizations, pre-immune serum and vaginal lavage samples were taken from each mouse, and the same samples were collected after the third (final) dose. Recombinant TdfJ was mixed with one of four adjuvants (alum, alum + MPL, Addavax, and
Emulsigen D) in 25 μg doses, and administered to female C57Bl/6 WT mice (eight mice per group, IP) in three-week intervals for a total of three doses. Roughly two weeks after the third dose, mice were vaginally challenged with \textit{N. gonorrhoeae} strain WHO-L to assess colonization/clearance. The following sample were collected: pre-immune serum and vaginal lavage, post-dose 1 and dose 2 serum, pre-challenge serum and vaginal lavage, post-challenge serum, vaginal lavage, nasal lavage. Samples from the hybrid antigen mice were shipped to me for characterization, while the adjuvant study samples remained in Toronto.

\textbf{D. ELISAs with Mouse Serum and Vaginal Lavages}

We first sought to assess whether immunization with the TdfJ/VcSLP hybrids induced antibodies that were cross-reactive against whole TdfJ, as was seen in previous hybrid studies (336, 423). We grew gonococcal cells expressing TdfJ and suspended them to a standardized density in PBS, then loaded them into a 384-well plate, which was left to dry such that the cells could adsorb. After blocking, we probed the TdfJ-expressing cells with a dilution series of pre- and post-immune sera and lavages from individual mice, and probed for TdfJ-specific IgG with anti-mouse-IgG secondaries (Figure 25). We found that none of the pre-immune samples (sera or lavages) contained any detectable TdfJ-specific IgG, and neither did the adjuvant-only or VcSLP-only samples. However, each of the five hybrid antigens contained cross-reactive IgG in the post-immune serum, with varying levels of response. Generally, signal from the TdfJ loops 5,6,11,2 hybrid was lower than others, although one mouse in this group was consistently higher. The highest serum IgG titer was seen in the loops 3,4,9,10 mice. IgG presence in vaginal lavages was not consistent with that
Gonococcal cells expressing whole TdfJ were resuspended in PBS and added to a 384-well plate, which was left to dry. After blocking, the plate was probed with a dilution series of pre-and post-immune mouse serum or vaginal lavage from individual hybrid-immunized mice (4 mice per group except 2 mice for VcSLP and Alum only). IgG that recognized whole TdfJ was detected by addition of HRP-conjugated anti-mouse-IgG secondaries, which were developed by addition of TMB and neutralized by 180 mM H$_2$SO$_4$. Signal was quantified by reading absorbance at 450 nm. Two experiments were conducted in an identical manner, and the results for experiment 1 are shown. Experiment 2 gave similar results.
seen in serum. The only hybrids that generated vaginal IgG were the loops 3,4,9,10 and 2,3,7,8 hybrids, which share loop 3 in common.

We also assessed presence of serum and vaginal IgA using a similar approach. Unfortunately, sufficient sample to investigate this in individual mice was not available after previous assays, so sera and lavages from each group were pooled instead. We again saw minimal signal from the pre-immune samples and the adjuvant/VcSLP-only samples, but did detect some serum IgA in the 3,4,9,10 / 5,6,11,2 / and 2,5,7,11 hybrids, and vaginal IgA from 3,4,9,10 and 5,6,11,2 (Figure 26). Notably, the loops 3,4,9,10 antigen generated anti-TdfJ IgG and IgA in both the serum and lavages, suggesting it to be a broadly immunogenic antigen.

**E. Functional Characterization of Mouse Serum and Lavages**

After demonstrating that hybrid immunization was indeed capable of eliciting a cross-reactive antibody response against TdfJ, we next turned our attention to the functional capabilities of those antibodies. This primarily involved two questions: 1) Can serum or lavage presence block S100A7 binding to TdfJ? 2) Can serum or lavage presence inhibit TdfJ-dependent S100A7 utilization? We first assessed whether S100A7 blocking was possible. To do this, we grew TdfJ-expressing gonococci and immobilized cell suspensions on nitrocellulose via a dot-blotter. After blocking to prevent nonspecific interactions, we incubated individual dots with a 3% solution of pooled pre- or post-immune serum or lavage samples, followed by probing with S100A7-HRP. We utilized no competitor as a negative control and excess unlabeled S100A7 as a positive. Upon signal development, we observed that no detectable deviations in S100A7 binding were present in the serum/lavage samples...
Figure 26. Mice immunized with TdfJ hybrids produce cross-reactive IgA

Gonococcal cells expressing whole TdfJ were resuspended in PBS and added to a 96-well plate, which was left to dry. After blocking, the plate was probed with pooled serum or vaginal lavage samples from hybrid-immunized mice. IgA that recognized whole TdfJ was detected by addition of HRP-conjugated anti-mouse-IgA secondaries, which were developed by addition of TMB and neutralized by 180 mM H₂SO₄. Signal was quantified by reading absorbance at 450 nm. N=2.
(Figure 27). To investigate this further, and in a more quantifiable way, we utilized an S100A7-binding ELISA with a similar setup. TdfJ-expressing cells were seeded into a microplate, blocked with BSA, then blocked with serum or lavage as described above, before being probed with S100A7-HRP (Figure 28). As seen in the dot blots, no binding inhibition was detected.

We also assessed whether serum or lavage presence could affect utilization of S100A7 as a zinc source. We grew strain RSC010 in CDM to induce zinc starvation, then seeded cultures of a standardized density into a 96-well plate that have been pre-treated with S100A7 as a zinc source, IPTG to induce tdfJ expression, and a 2% final solution of pooled serum (Figure 29) or lavage (Figure 30) sample. We assessed growth by monitoring OD\(_{600}\) over 12 hours, and our assay revealed that S100A7 utilization can indeed be inhibited by heat-inactivated serum or lavage presence, despite no blocking phenotype being apparent in the earlier assays. For serum, cultures given free zinc, S100A7 alone, or S100A7 and pre-immune serum grew without hindrance, but those treated with samples from the loops 2,3,7,8 and 3,4,9,10 hybrids were indistinguishable from those given no S100A7 at all (TPEN-only). Curiously, serum from the alum-only mice also inhibited growth, but we have not yet tested explanations for this. For the lavage-treated groups, again those with free zinc or S100A7 alone grew unencumbered, but strangely the pre-immune lavages curtailed growth altogether. As with the alum-only anomaly in the serum test, reasons for this have not yet been explored as of this writing. Furthermore, the two best-performing samples from the serum test (3,4,9,10 and 2,3,7,8) were, paradoxically, not distinguishable from the samples with free zinc or S100A7 alone, while all others showed a slight growth defect.
Figure 27. Dot blot to assess whether serum or lavage samples can block S100A7 binding by TdfJ

Gonococcal cells expressing TdfJ were dotted to nitrocellulose as described previously. After blocking, individual dots were incubated with a 3% solution of serum or lavage in blocker, then probed with S100A7-HRP. HRP signal was developed by addition of DAB substrate. Samples with no competitor, and samples with 10-fold molar excess of unlabeled S100A7 are shown as positive and negative controls, respectively. Blot is representative on N=2.
Figure 28. ELISA to assess whether serum or lavage samples can block S100A7 binding by TdfJ

Gonococcal cells expressing TdfJ were added to a 96-well plate as described previously. After blocking, individual wells were incubated with a 3% solution of serum or lavage in blocker, then probed with S100A7-HRP. HRP signal was developed by addition of TMB substrate and neutralized by 180 mM H$_2$SO$_4$ before being quantified by reading $A_{450}$. Samples with no competitor, and samples with 10-fold molar excess of unlabeled S100A7 are shown as positive and negative controls, respectively.
Gonococcal strain RSC010 was grown in 96-well plates in CDM supplemented with S100A7 as a sole zinc source, along with 2% serum from hybrid-immunized mice that was heat-inactivated prior to use. Growth was assessed by measuring OD$_{600}$ over 12 h. A few cultures started to clump at the bottom of the wells due to cell death after 9 h, which gave anomalous OD readings. Therefore, the graph was cut off at 9 h. Samples with free zinc and S100A7 alone are sown as positive controls, and samples with no zinc (TPEN) as a negative. N=2.
Figure 30. Presence of vaginal lavages differentially inhibits S100A7 utilization

Gonococcal strain RSC010 was grown in 96-well plates in CDM supplemented with S100A7 as a sole zinc source, along with 2% vaginal lavages from hybrid-immunized mice. Growth was assessed by measuring OD$_{600}$ over 12 h. Samples with free zinc and S100A7 alone are sown as positive controls, and samples with no zinc (TPEN) as a negative. N=2.
F. Adjuvant Impacts on TdfJ-based Vaccination

Mice immunized with TdfJ that was formulated with different antigens were the subjects of experimental vaginal infections to assess adjuvant-dependent differences in disease/colonization outcome. Two weeks after their final vaccine dose, mice were vaginally infected with *N. gonorrhoeae* strain WHO-L, which was selected because its *tdfJ* gene is a 100% match for the one used to generate the recombinant antigen. After day 0 of infection, vaginal swabs from each mouse were collected daily and plated for enumeration of gonococcal CFU to elucidate what percentage of mice were still colonized (Figure 31). This experiment revealed differential colonization lengths that varied in an adjuvant-dependent manner. Naïve mice were colonized for 20 days post-infection and did not reach 50% clearance until day 13. Mice immunized with alum-based formulations (+/- MPL) were colonized for similar overall lengths of time, and indeed alum without MPL generated 50% clearance slower than seen for naïve mice (day 13 vs. day 14). Alum + MPL generated 50% clearance on day 9. The Addavax and Emulsigen D formulations generally cleared infection more quickly than the others, though 10% of Addavax mice stayed colonized until day 19. However, the Addavax mice passed 50% clearance by day 5, with the Emulsigen D mice following on day 6, and strikingly, all Emulsigen D mice had fully cleared infection by day 14, faster than any other group.

Post-dose 1, 2, and 3 serum samples from these mice were also assessed for presence of TdfJ-specific IgG in a manner similar to that described for the TdfJ hybrids, the results of which are shown in Figure 32. After a single dose, minimal IgG were observed, but detection increased considerably after doses 2 and 3. Strikingly, Emulsigen D formulations
Figure 31. TdfJ formulated with different adjuvants confers variable protection against genital gonococcal challenge

Groups of eight female C57Bl/6 WT mice were immunized three times at three-week intervals with TdfJ formulated with one of four adjuvants. Two weeks after their third dose, mice were vaginally challenged with \textit{N. gonorrhoeae} strain WHO-L, after which point daily vaginal washes were plated for enumeration of CFU. The graph shows the percentage of mice that were culture positive as a function of time passed since infection. This figure was generated by Elissa Currie, University of Toronto.
Gonococcal cells expressing TdfJ were resuspended as previously described and seeded into a 96-well plate. Following adsorption, plates were blocked and subsequently probed with a 1:5000 dilution of serum from mice immunized with TdfJ in variable adjuvant formulations. TdfJ-specific IgG was detected by addition of anti-mouse-IgG secondaries, and IgG levels are shown as a function of dose number and adjuvant type. Figure generated by Elissa Currie, University of Toronto.

Figure 32. TdfJ-specific IgG titers vary according to vaccine adjuvant
generated low TdfJ-specific IgG over the duration of immunization, which stands in direct contrast to the observation that it was the most protective adjuvant tested. In conjunction with this observation, the second-lowest IgG detected was with the Addavax formulation, which was also in the more-protective group in the colonization experiments. Collectively, these two assays demonstrated an inverse relationship between overall antibody titers and protection, suggesting that antibody presence alone is not necessarily predictive of protection, and vice-versa.

III. Discussion

In this chapter, we performed initial characterization of gonococcal TdfJ as a vaccine antigen with promising results. In one arm of this study, we showed that TdfJ loops fused to a lipoprotein scaffold can elicit TdfJ-specific antibodies with various functional capabilities, and in the second, we showed that formulating recombinant TdfJ with different adjuvants produces differential outcomes with regard to gonococcal colonization and antibody production. Nevertheless, certain limitations and omissions from the current dataset must be discussed when framing these results in the context of the field as a whole.

The hybrid antigen platform was initially envisaged to relieve the difficulties in purifying and working with integral membrane proteins, and as discussed, it has been successful in other studies (336, 423). However, in our hands, the current iterations of the antigens are not suited to traditional purification or soluble stability. The net result of this deficiency was the requirement that we either forge ahead with unfolded, solubilized inclusion body preps, or re-engineer the antigens with single loops or as MBP-fusions, both of which yielded stable antigens in the cited studies. We elected the former, operating on
the hypothesis that MHC presentation of peptide antigens would require protein digestion anyway, and this appeared to be a sound decision – immunization with these antigens generated cross-reactive antibodies with biologically-relevant function. Regardless, appraisal of the overall utility of this platform, especially as concerns the presumed ease of use and production scalability, seems warranted.

Our TdfJ hybrids ubiquitously produced serum IgG, and both hybrids expressing the loop three peptide induced vaginal IgG as well. Interestingly, this observation is consistent with early characterization of ZnuD by Hubert et al., who noted that serum from convalescent meningitis patients showed consistently high reactivity against loop 3 peptides, suggesting it to be a particularly immunogenic region (406). Furthermore, serum from both loop 3-bearing antigens showed the capacity to inhibit S100A7 utilization, and we know from the information presented in chapter 4 that loop 3 contains a critical motif, the α-helix, involved in S100A7 binding and utilization. It is noteworthy that the helical region in question is part of the loop 3 peptide antigen, so some antibody shielding of that motif seems plausible. Despite S100A7 use inhibition, however, we paradoxically did not observe any blocking of binding. While not directly tested, we currently hypothesize that this is a result of excessively dilute TdfJ-specific antibodies in our samples, and that some minor optimization of the experimental setup may address this.

Regarding the adjuvant study, we note that antibody titers are not predictive of protection and vice-versa, and indeed this concurs with previous studies which showed that TbpA, while potentially protective, induces a paucity of antibody response (334, 422). Curiously, however, antibody presence even at low numbers appears to correlate to
protection against gonococcal infection. A recent study by Liu et al. demonstrated that a
Th1-biased immune response in mice, induced by exogenous administration of IL-12,
protected mice from gonococcal colonization (358). Critically, this response was noted for
the presence of gonococcus-specific IgG and IgA, both in serum and vaginal washes, and
protection was lost in B-cell-deficient mice. When considering these data together, it seems
likely that antibody presence in some capacity is a requirement, and that an effective
antibody response performs some specific, although as-yet-undetermined function. For the
meningococcus, a known correlate of protection is presence of serum bactericidal
antibodies, an observation that traces back to as early as 1922 when George Heist and
colleagues noted that meningococci isolated from infected patients were more resistant to
killing in whole blood compared to meningococci from healthy carriers (432). Today,
subsequent studies following the advent of meningococcal vaccination have confirmed the
importance of bactericidal antibodies in protection against invasive meningococcal disease
(433). It is reasonable therefore to hypothesize that this phenomenon may hold true for the
gonococcus, but recent reports have indicated that no correlation between bactericidal
antibodies and protection against gonococcal urethritis exists (434), though these studies
did not assess effects on disseminated disease.

While the primary mechanism by which antibodies protect against gonococcal
infection is not yet clear, our adjuvant study clearly demonstrated that adjuvant selection is
a key driver of a protective response, suggesting that adjuvant-dependent polarization of
the immune response is a worthwhile consideration. Alum is known to skew the immune
response towards a Th2 bias, resulting in higher levels of IgG1 and limited numbers of IgG2
subtypes (435). Interestingly, such a response in mice has been shown to limit activation of the classical complement pathway through IgG1-mediated inhibition of C1q binding by other IgG subtypes (436). Addavax is similar to another adjuvant, MF59, which is an oil-in-water emulsion licensed for use in various influenza vaccines. MF59-adjuvanted vaccines have traditionally induced a Th2-biased response, with a trend of eliciting higher antigen-specific antibody production and better protection in mouse models compared to alum (437). This stands in partial contrast to our results, which showed a larger antibody response from alum, but it must be noted that MF59 has primarily been used for viral vaccines, so some inconsistency is perhaps unsurprising. Finally, little is published about Emulsigen D other than its common use in veterinary vaccines, but one study utilizing it as vaccine adjuvant against *Streptococcus suis* noted that it induced a limited humoral response that was still protective, with a mixed IgG1/IgG2 response, which agrees with our results (438).

Certain omissions from our current dataset make the two studies difficult to reconcile with each other, with the most notable exception being the lack of any protection studies with the TdfJ hybrids. As of this writing, sufficient antisera and lavage samples from both the hybrid and adjuvant studies remain available for a full workup of antibody characterization tests, and it is anticipated that future work will fully evaluate both sets for isotype, S100A7 blocking/inhibition, bactericidal activity, opsonization, ADCC, etc. If a future study were to re-evaluate the hybrids for their protective capabilities, one could then cross-reference the two “complete” studies to identify which antibody characteristics, if any, correlate with protection in the mouse model. One must consider, however, that certain
limitations in the current mouse model still persist, namely that human-specific cell-surface receptors and iron/zinc sources are not present in WT mice, and therefore experimental gonococcal infections do not necessarily mirror natural infection in humans (380). This is an especially large obstacle in evaluating the potential relevance of S100A7 blocking/inhibition by vaccination, as this mechanism is not currently a factor in the protection seen in our WT mice; TdfJ does not interact with mouse S100A7 (249). In light of this, it may be prudent to await the completion of a human S100A7-transgenic mouse, which is currently in development, before returning to such experiments.

Author’s Note: The data presented in chapter 5 are the result of a joint effort between myself and members of the laboratories of Drs. Tony Schryvers, Scott Gray-Owen, and Trevor Moraes, all of whom made significant contributions to its progress thus far. The opinions reflected in the results and discussion sections of this chapter are my own and do not necessarily reflect those of my colleagues. Any errors or omissions, likewise, are my own.
CHAPTER 6: SUMMARY AND PERSPECTIVES

It is no overstatement that gonorrhea poses a serious threat to global health. This human-specific disease afflicts millions each year, elicits no protective immunity, is not currently preventable by vaccination, and has demonstrated resistance to every class of antibiotic currently available (60, 73, 76, 387). The need for novel therapeutic and prophylactic measures is clear, and a recurring subject of interest as a vaccine target are the outer-membrane TonB-dependent transporters, which facilitate acquisition of iron and zinc during infection (413).

In these studies, we focused our attention on gonococcal TdfJ, a TdT that is ubiquitously expressed among all known Neisseria species. This transporter, which was previously shown to facilitate uptake of free zinc (287, 291), proved to be highly-conserved across the genus, suggesting a role in not only pathogenesis, but overall survival for the Neisseria. Further, we identified that TdfJ function is not restricted to uptake of free zinc, but that it also directly co-opts the innate immunity protein S100A7 for zinc piracy. In doing so, we have now identified ligands for six of the eight gonococcal TdTs, with TdfF and TdfG still outstanding. Structural studies with the gonococcal TdTs have revealed the conservation of a key extracellular motif, the loop three α-helix. This feature is most well studied in TbpA, where it is known to interact with the iron-coordinating cleft in the C-lobe of transferrin to liberate iron (252, 253). Two subsequent reports demonstrated that mutagenesis of this helix was sufficient to either inhibit or altogether abolish transferrin binding and iron piracy. In the first, Cash et al. (254) primarily relied on alanine scanning within the helix to assess the impacts of mutagenesis, and found that while individual
missense mutations were deleterious, no single mutation fully abrogated the transferrin interaction, which was seen upon deletion of, or HA epitope insertion into the helix.

These results stimulated the hypothesis that the amino acid sequence of the helix may not be as important as the actual helical shape, which was subsequently tested by a series of proline insertions (439). As a planar, cyclic amino acid, proline possesses multiple dihedral angles which are locked, thus limiting flexibility (415), making it a promising choice to physically disrupt an α-helix. Ultimately, this investigation revealed that two helix residues, D355 and A356, were critical to transferrin binding and iron piracy; the D355P and A356P mutants were significantly attenuated for these functions. These findings led us to proline insertion in the TdfJ α-helix, which we hypothesized participated in zinc piracy from S100A7 not only because of helix presence, but because of its localization between putative zinc-sensing clusters (292). Indeed, proline insertion at two key residues, K261 and R262, completely abolished interaction with S100A7, strengthening the hypothesis that the extracellular helix is a conserved evolutionary adaptation. Future efforts will likely aim to assess the importance of this motif in other gonococcal TdTs, many of which are known or predicted to contain it (440).

Beyond structure/function analysis, we also evaluated TdfJ as a vaccine antigen in two platforms: whole recombinant protein with multiple adjuvant formulations, and as a hybrid antigen presenting putatively immunogenic loop peptides. These studies were successful in illuminating the importance of adjuvant selection in vaccine development, and also highlighted the paradoxical relationship between antibody presence and protection from urogenital colonization, wherein high titers are not associated with protection. While
Outright antibody presence is apparently not a contributing factor, the literature suggests an important, if undefined, functional role for the antibodies that are present. Promisingly, immunization with TdfJ hybrids generated cross-reactive antibodies with identifiable functions, with the expectation that others will be established in the future. However, as discussed, there are currently obstacles in drawing relationships between the two studies. Discussions about experimental vaccinations in this document have also largely been restricted to humoral immunity, with little attention given to cell-mediated responses. While *N. gonorrhoeae* is primarily an extracellular pathogen, it is capable of facultative intracellular survival, and there is evidence that infected humans develop cellular immune responses to certain gonococcal antigens. Early evidence to this end measured lymphocyte proliferation after antigen stimulation or as a comparison between infected and uninfected human volunteers (441, 442). These studies showed increased antigen-dependent proliferation in infected individuals compared to control subjects with no history of gonorrhea. However, the specific gonococcal antigens recognized by the cellular response are not well understood, and to date only two antigens have received appreciable attention in this area. In one study, Tsirpouchtsidis *et al.* observed activation of CD4+ and CD8+ T-cells, and CD56+ NK cells, in response to treating uninfected humans with recombinant IgA1 protease (443). This was accompanied by IL-4, IL-10, IFN-γ, and TNF-α release in peripheral blood mononuclear cells. Another study assessed the response to PorB, as previous studies showed an association between lymphocyte proliferation and incubation with PorB in infected patients. This report showed a significant increase in IL-4-secreting CD4+ T-cells, but no measurable increase in IL-2, IL-12, IFN-γ, or TNF-α (444). While mucosal infections are
not typically associated with a concomitant change in circulating cytokine levels, the
capacity for the gonococcus to drive elevated IL-17 and IL-23 levels is well-established and
has been discussed in earlier sections (390, 392, 445). Finally, the role of cytotoxic CD8+ T-
cells and NK cells is largely unexplored, with most data only available in the context of
patients co-infected with HIV or CMV, wherein N. gonorrhoeae presence appears to
correlate with fluctuation of CD8+ T-cell activation (446, 447). There are currently no known
relationships between the TdTs and cellular immunity.

Together, our observations about TdfJ as a vaccine antigen coupled with what is
known about the human immune response to gonococcal infection generates two lingering,
and rather important questions: 1) Because no correlates of protection have been
confirmed, what kind of immune response should TdfJ-based vaccination strive for to elicit
protection? 2) Since gonococcal infection is known to dampen the adaptive immune
response, how might vaccination overcome or prevent this? Traditional wisdom suggests
that humoral, rather than cell-mediated immunity would be critical for a primarily
extracellular infection. Indeed, this observation is supported by mouse studies which
revealed that IgG and/or IgA are necessary for immunity to N. gonorrhoeae (357), and
further bolstered by observations that presence of bactericidal antibodies was associated
with reduced incidence of salpingitis (448). However, recent evidence demonstrated that
serum antibody-mediated bacteriolysis, while ostensibly important to control
dissemination, is not associated with protection against gonococcal urethritis (434), and it
has long been understood that natural infection drives a broadly-reactive and minimally-
gonococcus-specific IgM response (449). Nevertheless, induction of circulating and/or
mucosal IgG/IgA appears desirable and may be accomplished through a combination of adjuvant choice and immunization route. Our own adjuvant studies will in the future be analyzed with sufficient granularity to establish the ratio of Ig isotype and subtype, as well as spatial distribution, induced by our adjuvant panel, lending useful complementary data to what we have already observed regarding protection. Beyond these, however, certain adjuvants are well studied regarding such characteristics. For example, Cholera toxin (CT) and *E. coli* heat-labile toxins are known potent mucosal adjuvants and have been well-characterized during nasal and vaginal administration (450). Indeed, vaccines adjuvanted with CTB induce vaginal antibodies (451), though use of such an adjuvant in humans poses the significant danger of vaccine trafficking to the central nervous system through the olfactory nerve (452). Beyond these, IL-12 has shown promise as a gonococcal vaccine antigen in mice by virtue of induced Th1 proliferation and antibody induction (358), and has similarly shown promise against HIV when administered intranasally alongside IL-18 and IL-1 (453). Beyond adjuvants, immunization route is a worthwhile consideration, especially as secretory IgA seems to be an appealing counter to a mucosal infection. Intranasal vaccination is an effective route for inducing antibodies in the genital tract (454, 455), so such a route coupled with a potent antigen could be a promising path towards an antibody-centric, mucosal immune response. Of course, the desirability of such a response, while logical, requires further understanding of antibody-dependent protection against gonorrhea and a knowledge of the respective roles of IgA and IgG.

Beyond this, a Th cell response is presumably required to establish the required antibody response and immunological memory; induced Th1 stimulation has been shown to
sidestep certain aspects of gonococcal immune suppression. To that end, certain characteristics of the *N. gonorrhoeae*-dependent dampening of adaptive immunity are known, though their mechanisms are not yet entirely clear, and these could be exploited by a vaccine which specifically inhibits them to allow memory to develop. Many of the known immunomodulatory efforts of the gonococcus, such as IL-10-, TGF-β-, and type 1 Treg-dependent suppression of Th1 and Th2 development (107, 456) have already been discussed in this document, but certain others may also be promising areas for evaluation. For example, the gonococcus induces NLRP3-dependent apoptosis of antigen presenting cells (457) and, with respect to putatively necessary antibody generation, Opa proteins binding host CEACAMs have been reported to inhibit B-cell proliferation (105). It is therefore feasible that a vaccine designed with these mechanisms in mind may preclude them in a targeted way, allowing memory to develop. The TdTs may be promising antigens to serve as a basis for such efforts.

The *Neisseria* are not alone in their utilization of TdTs, and indeed these receptors in numerous other pathogens have been considered promising drug and/or vaccine targets as well. The TdTs meet most of the characteristics one would associate with a strong vaccine target: 1) surface exposure, 2) important for pathogenesis, 3) inducible expression during infection, 4) ubiquitous among pathogenic strains, and 5) ability to induce a protective response. To begin, as outer-membrane proteins the TdTs have numerous surface-accessible loops, which are common immune targets (458, 459). We have already discussed at length the immunogenicity of gonococcal TdTs, especially TbpA and TdfJ loops, but this is a common outcome in other species as well. For example, TdTs of *A. baumannii* are known
to elicit antibodies which form immune complexes and facilitate phagocytosis (460).

Similarly, previous chapters discussed the *Neisseria* TdTs regarding their roles in pathogenesis, but similar importance has been observed for species such as *Salmonella enterica*, *Francisella tularensis*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and others (461-465). Lastly, owing to the nutrient-scarce conditions presented in the host, expression during infection is common amongst TdT variants (248, 466).

For these reasons, it is unsurprising that vaccine efforts targeting TdTs are plentiful and various approaches have been utilized, ranging from subunit vaccines to attenuated strains. For subunit vaccines, a highlight is the effort to prevent infection by Uropathogenic *E. coli*, one of the most prevalent pathogens associated with urinary tract infections. Individual studies targeting four TdTs – IreA, Hma, FyuA, and IutA – used nasal immunizations adjuvanted with cholera toxin and showed protection in a mouse model (467-470). Beyond their individual use, these four antigens were also combined into a subunit vaccine which elicited high titers of protective antibodies (471). *P. aeruginosa*, a nosocomial pathogen responsible for chronic respiratory infections, also saw reduced colonization after nasal immunization with a vaccine comprised of FpvA peptides fused to KLH, an approach not dissimilar to the hybrid antigen platform discussed in chapter 5 (472). Attenuated bacterial strains have also been used for species including *S. enterica* and *Shigella dysenteriae*. For *S. enterica*, a *fepA-cir-IroN* triple mutant was significantly attenuated in mouse infection, and no immunized mice were killed upon administration of challenges at lethal doses (461). For *Shigella*, a quadruple mutant (*Δent Δfep Δics ΔstxA*) was used in a phase I clinical trial in humans, in which it was well tolerated and induced high
levels of IgA and circulating IgG (473). Despite these promising results, however, certain drawbacks must be considered, namely antigenic variation and systematic redundancy. Chapter 1 discussed two mechanisms of high-frequency antigenic and phase variation prevalent in the *Neisseria* (325, 474), but additional appreciation must be offered to the fact that, as surface-accessible antigens, the TdTs are subject to considerable selective pressure, which is likely to also drive variation. Furthermore, as multiple TdTs which import the same cargo (iron, zinc, siderophores, vitamins, etc.) can exist within a single genome, there is potential for redundancy between these systems, such that targeting a single TdT may be insufficient. This was exemplified by Anderson et al. (272), who demonstrated that utilization of lactoferrin was sufficient to overcome the defect imposed by absence of a functional transferrin receptor in *N. gonorrhoeae*. It is therefore likely that multiple TdTs must be targeted to effectively establish long-term protection.

In conclusion, a brief word about the future prospects of a potential gonococcal vaccine: Beyond the bench work and clinical trials, consideration must be given to exactly where, when, and how a vaccine will be introduced into the population. After all, the most protective vaccine in the world is wasted if it doesn’t reach the people who need it. Although the exact nature of an eventual gonorrhea vaccine rollout is a problem for the future, current efforts have offered some predictive insight into the vaccine’s population-level impacts. A recent report from Hui et al. (475) utilized individual-based, site-specific mathematical modeling to simulate the introduction of a gonorrhea vaccine and predict *N. gonorrhoeae* transmission in a population of 10,000 men who have sex with men (MSM). The model predicted that a 100% protective vaccine administered to 30% of the MSM
population could decrease disease incidence by 94% within two years. For a 50% protective vaccine, incidence was predicted to fall by 62% assuming the same rate of vaccination. Further, gonorrhea was predicted to be eliminated within eight years with a vaccine of ≥50% efficacy lasting 2 years, provided boosters were available. This study suggests that a vaccine of only modest efficacy could rapidly and substantially reduce gonorrhea prevalence with a rational immunization strategy. To that end, the concept of immunizing against sexually-transmitted infections is not new, but the nature of these infections as a consequence of sexual activity presents an added obstacle in a climate already rife with vaccine hesitancy. Young adolescents, pre-sexual debut, are presumed to be likely targets for such a vaccine, and it is unclear the extent to which parents will agree to their children being immunized against STIs. One qualitative study from 2004 addressed this question via interviews with 34 parents regarding their opinions on accepting adolescent vaccination against herpes, HPV, HIV, and gonorrhea (476). Promisingly, most parents (>70%) viewed all four vaccines as acceptable, frequently citing the desire to protect their children and concerns about potential outcomes of the disease(s). While this study’s age may mean that its conclusions do not hold true today, vaccine acceptance is still a noteworthy topic, and tools such as the WHO Vaccine Hesitancy Scale are in use to assess current and future vaccine prospects (477).
CHAPTER 7: MATERIALS AND METHODS

A list of bacterial strains and their genotypes can be found in Table 3. All plasmids and their descriptions are available in Table 4. All oligonucleotides and their sequences are in Table 5.

I. Bacterial Strains and Growth Conditions

*E. coli* strains were cultured in Luria-Bertani (LB) medium supplemented with antibiotics at the following concentrations: 100 µg/mL for carbenicillin, 34 µg/mL for chloramphenicol, and 50 µg/mL for spectinomycin. Strains of *N. gonorrhoeae* were maintained on GC medium base (Difco) agar with Kellogg’s supplement I (478) and 12 µM Fe(NO₃)₃ (GCB plates) at 36°C at 5% CO₂ with shaking at 225 RPM. For growth of *N. gonorrhoeae* in liquid culture, both rich and defined media were used, and zinc restriction was accomplished by addition of TPEN (Sigma) at 10 µM for GC broth, or 1 µM for defined medium. For growth in defined medium, colonies from GCB plates were used to inoculate chemically defined medium (CDM) that had been treated with Chelex-100 resin (Bio-Rad). These cultures were grown as described until log phase before back dilution and treatment with 24 µM Fe(NO₃)₃, 5 µM S100A7, 10 µM ZnSO₄, and/or 1 or 5 µM TPEN for preparation of whole cell lysates (WCLs), or treated with growth premix (described below) for zinc-dependent growth assays. For WCLs, growth was allowed to proceed for 6 hours as described before lysates of a standardized density were collected and subjected to SDS-PAGE and western analysis.
### Table 3. Strains Used in This Study

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**Table 4. Plasmids Used in This Study**

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<tr>
<td>oGSU023</td>
<td>GACGGAGCTCGAATTTCAAAATTTCACGTTCGCGC</td>
<td>tdfJ expression vector - R</td>
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<tr>
<td>oGSU120</td>
<td>AACGGGCAGACGGATTAAAG</td>
<td>tdfJ sequencing</td>
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<tr>
<td>oGSU121</td>
<td>GGCACCCAGGCTTTACACT</td>
<td>VcSLP hybrids - F</td>
</tr>
<tr>
<td>oGSU122</td>
<td>GCCAGTGTGACTCTAGTGGACGCG</td>
<td>VcSLP hybrids - R</td>
</tr>
<tr>
<td>oGSU285</td>
<td>ACGACGACAAGCATATGACCTTGAATCAGATGATCGGC</td>
<td>tdfJ hybrid 2,5,7,11 into pET-19b - F</td>
</tr>
</tbody>
</table>
oGSU286  GTTAGCAGCCGGATCTTACTCTTCTTGTGCTGAGAAAACC  \textit{tdfJ} hybrid 2,5,7,11

into pET-19b - R

oGSU287  ACGACGACAAGCATATGAACTTAGAAAGCCAGATGATCGG  \textit{tdfJ} hybrid 2,3,7,8

into pET-19b - F

oSGU288  GTTAGCAGCCGGATCTTACTCTTACTTCGCGCTG  \textit{tdfJ} hybrid 2,3,7,8

into pET-19b - R

oSGU289  ACGACGACAAGCATATGAACTTAGAAAGCCAGATGATCGG  \textit{tdfJ} hybrid 4,5,10,11

into pET-19b - F

oGSU290  GTTAGCAGCCGGATCTTACTCTTCTTACTTCGCGCTG  \textit{tdfJ} hybrid 4,5,10,11

into pET-19b - R

oGSU291  ACGACGACAAGCATATGAACTTAGAAAGCCAGATGATCGG  \textit{tdfJ} hybrid 5,6,11,2

into pET-19b - F

oGSU292  GTTAGCAGCCGGATCTTACTCTTCTTACTTCGCGCTG  \textit{tdfJ} hybrid 5,6,11,2

into pET-19b - R

oGSU293  ACGACGACAAGCATATGAACTTAGAAAGCCAGATGATCGG  \textit{tdfJ} hybrid 3,4,9,10

into pET-19b - F

oGSU294  GTTAGCAGCCGGATCTTACTCTTCTTACTTCGCGCTG  \textit{tdfJ} hybrid 3,4,9,10

into pET-19b - R
II. Gonococcal Mutant Construction

To construct the ectopic tdfJ mutants, mutated tdfJ gene sequences were submitted to either Bio Basic Inc. or GENEWIZ, Inc. for de novo synthesis, and subsequently cloned into pVCU234 between the XmaI and XhoI sites. The XmaI site was reconstructed during this cloning step, leaving six nucleotides between the plasmid’s ribosome binding site and the tdfJ start codon. For gonococcal transformations, plasmids were first linearized with PciI and then used to transform a piliated population of gonococcal strain MCV928 (402). Transformants were recovered on GCB plates supplemented with 1 μg/mL chloramphenicol, and presence of the tdfJ gene(s) was verified by PCR.

III. Construction of Expression Plasmids

To generate expression systems for recombinant TdfJ, the mature coding region of WT and mutant tdfJ was amplified from the complementation plasmids by PCR, and fragments were subsequently cloned via In-Fusion (Clontech) into pET-20bHT (479) between the NcoI and XhoI sites. pET-20bHT contains a pelB signal sequence followed by a 10X N-terminal His tag and a TEV cleavage site. Clones were used to transform E. coli, which were recovered on 100 μg/mL carbenicillin and verified by PCR and sequencing. For hybrid antigen expression plasmids, the tdfJ/VcSLP hybrid genes were amplified from their e5460 plasmids via PCR and ligated into pET-19b between the Ndel and BamHI sites via In-Fusion. Ligation reactions were used to transform DH5α E. coli, which were selected on 100 μg/mL carbenicillin and confirmed by sequencing.
IV. Purification of S100A7, S100A12, TdfH, and TdfJ

S100A12 was produced from a pGEMEX expression vector provided by Professor Claus Heizmann and purified as described elsewhere (233). S100A7 was produced from a pET-22b expression vector provided by Professor Joachim Grötzinger (pET-22b-pso) using the protocol described by Grötzinger and coworkers (480). His-tagged TdfH was purified as described elsewhere, with the tag cleavage step omitted (288). Wild-type and mutant TdfJ were produced from C41 (DE3) E. coli. For purification, a starter culture of appropriate plasmid was prepared in Luria-Bertani (LB) medium supplemented with 100 μg/mL carbenicillin and was sub-cultured into Terrific Broth (TB) with the same antibiotic. Expression cultures were grown without inducer for approximately 48 h at 20°C, and cell pellets were harvested by centrifugation (10,000 x g for 1 h, 4°C). Cell pellets were resuspended in lysis buffer (20 mM HEPES pH 8.0, 250 mM NaCl, 100 μg/mL lysozyme, 1 mM phenylmethylsulfonyl fluoride [PMSF]) using a Dounce homogenizer, with 10 mL buffer used per gram of cell paste. Cell suspensions were then mechanically lysed via two passages through an Emulsiflex C3 (Avestin, Inc.) with homogenizing pressure of ~17,500 PSI. Insoluble material was removed via centrifugation (30,000 x g for 30 minutes, 4°C) and the supernatant was collected and mixed with 1% Triton X-100 for 1 h at room temperature. The supernatant was then centrifuged at 160,000 x g for 1 h to pellet membranes, and remaining supernatant was discarded. Membranes were resuspended in minimal volume of membrane buffer (20 mM HEPES pH 8.0, 250 mM NaCl, 10% Elugent, 1 mM PMSF) and allowed to mix at 4°C overnight. Undissolved membranes were then pelleted via centrifugation (12,500 x g for 20 minutes), and the remaining solubilized material was mixed
with Ni-NTA resin for 2-3 h at 4°C. The resin was collected in a chromatography column, then washed with 10 CV each of wash buffer (20 mM HEPES pH 8.0, 150 mM NaCl, 0.25% Elugent, 1 mM PMSF) containing 0, 50, 100, 200, and 300 mM imidazole. TdfJ eluted cleanly at 300 mM. Eluted protein was then dialyzed overnight at 4°C into PBS pH 8.0 + 0.25% Elugent. Following dialysis, if appropriate, protein sample were incubated overnight at 4°C with 1 μL TEV protease to cleave the 10X-His tag, followed by tag recapture via brief incubation with Ni-NTA resin. If necessary, proteins were then concentrated using a 50,000 MWCO centrifugal filter, then flash frozen for storage at -80°C.

V. Hybrid Antigen Inclusion Body Preparation

5mL starter cultures of C41 (DE3) E. coli housing hybrid antigen expression plasmids were grown overnight at 37°C with 100 μg/mL carbenicillin. The next day, these were subcultured into 50 mL Terrific Broth with the same antibiotic and grown until their OD

reached ~0.7-1.0. At this point, 1 mM IPTG was added to induce gene expression, which was allowed to proceed for 3-4 h. Cell pellets were then harvested via centrifugation at 10,000 x g for 10 minutes, and supernatant was discarded. Pellets were resuspended in 20 mL lysis buffer (PBS pH 8.0, 5 mM EDTA, 1 mM PMSF, 20 mM CaCl₂, 0.1 U/mL DNase 1, 100 ug/mL lysozyme) and dissolved with a Dounce homogenizer. Resuspended pellets were then lysed via three passages through an Emulsiflex C3, with homogenizing pressure of ~15,000 to 17,500 PSI. Lysed samples were centrifuged again at 12,500 x g for 20 minutes, and supernatant was discarded, leaving only insoluble material. These pellets were subsequently washed three times with cold PBS pH 8.0 + 1% Triton X-100 and 5 mM EDTA, followed by two additional washes with PBS pH 8.0 + 5mM EDTA only to leave behind the
pure inclusion body pellets. Inclusion bodies were then solubilized via addition of 10 mL buffer containing 8 M urea, 1 mM DTT, and 0.5% Sarkosyl and mixing at room temperature for 30-45 minutes. Solubilized preps were then dialyzed against two consecutive volumes of 2 L PBS pH 8.0 for 4 h each to remove urea, then aliquoted and flash frozen.

VI. Western Blotting

Whole-cell lysates of gonococci were harvested by pelleting cultures of a standardized optical density (100 Klett units in 1 mL culture) and resuspending cells in 2X Laemmli solubilizing buffer before storage at -20°C. Immediately preceding use, samples were thawed, mixed with 5% β-mercaptoethanol, and boiled for 5 minutes. Protein samples were separated on a pre-cast 4-20% gradient polyacrylamide gel before transfer to nitrocellulose. Blots were stained with Ponceau S to verify equal protein sample loading. To detect TdfJ, blots were first blocked in 5% (w/v) nonfat dry milk dissolved in TBST. Blots were then probed with either TdfJ peptide-specific guinea pig polyclonal antiserum (1:200 in blocker) or mAb hybridoma supernatant (1:100 in blocker) for 2 h at room temperature. Generation of the TdfJ peptide antiserum is described elsewhere (293). Blots were washed three timed with TBST and then probed with either HRP-conjugated anti-guinea pig-IgG or HRP anti-mouse IgG secondaries (1:3000 in blocker) for 1 h. Blots were then washed again and developed using SuperSignal West Femto Extended Duration Substrate (ThermoFisher) and imaged on a Bio-Rad ChemiDoc Gel Imaging System using 4x4 auto ECL detection.

VII. Metal Loading of Proteins and Production of Growth Mixes

Human transferrin (Sigma) was dissolved in a mixture of 40 mM Tris, 150 mM NaCl, and 10 mM NaHCO₃ at pH 8.4 (initial buffer) before being incubated with FeCl₂ to result in
~30% Fe saturation. After incubation, this solution was dialyzed against excess 40 mM Tris, 150 mM NaCl, and 20 mM NaHCO₃ at pH 7.4 (dialysis buffer) to remove unbound iron. Bovine apo-transferrin (Sigma) was dissolved in initial buffer and not treated with any metal before dialysis, such that it could sequester any residual iron in the final mix. S100 proteins were maintained in their own unique buffers after being purified as described. For zinc loading, the dimeric S100 proteins were incubated with ZnSO₄ at a 2:1 molar ratio (dimer:zinc) to accomplish ~25% zinc loading. These ingredients plus TPEN and phosphate buffered saline, pH 7.4 (PBS) were combined into a concentrated growth “premix” which was used to restrict *N. gonorrhoeae* to defined sources of iron and zinc for growth in microtiter dishes. When diluted by liquid culture, final concentrations in the premix were as follows: 7.5 μM 30%-Fe human transferrin, 3 μM bovine apo-transferrin, 5 μM 25%-Zn S100 protein, and 1 μM TPEN. For a positive zinc control, TPEN was omitted and S100 proteins were replaced with 5 μM ZnSO₄, and for a negative control all zinc sources were omitted.

VIII. Zinc Limited Growth Assays

Gonococcal growth assays in zinc-restricted conditions were performed as previously described (483). In short, gonococci were grown to exponential phase CDM to induce zinc stress. At this point, cultures were back diluted to OD₆₀₀ = 0.02 in the same medium and added into a 96-well plate, where they were supplemented with either 5 μM ZnSO₄ as a positive control, 5 μM TPEN as a negative control, or 5 μM TPEN plus 5 μM Zn-loaded S100A7 to test S100A7 utilization. 1mM IPTG was added where appropriate. For growth with antisera or lavages, 2 μL of either serum or lavage was added to 100 μL final
volume in the wells. Cultures were grown for 12 h (36°C, 5% CO₂, 225 RPM) in a Cytation5 plate reader (BioTek), with OD₆₀₀ measured every 30 minutes to track growth.

IX. Whole Cell Dot Blots

Gonococcal strains were grown on GCB agar plates with or without 1 mM IPTG before being resuspended in PBS. Cell suspensions were standardized to OD₆₀₀ = 1.0 before being dotted onto nitrocellulose in a dot blotter, then cells were allowed to adsorb and blots were left to dry. The dried blots were blocked with either 5% (w/v) bovine serum albumin (BSA) or 5% (w/v) nonfat dry milk dissolved in Tris-buffered saline plus 0.05% Tween 20 (TBST). For S100A7 binding assays, blots were returned to the dot blotter and probed with HRP-labeled S100A7 (S100A7-HRP) dissolved in blocker at the following concentrations: 0.4, 0.2, 0.1, 0.05, 0.025, and 0.0125 μM. After probing for 1 h at room temperature, probe was removed via vacuum and the blot was washed three times for ten minutes using TBST. HRP signal was developed using CN/DAB substrate (ThermoFisher). For monoclonal antibody probing during mAb characterization, dried and blocked blots were returned to the blotter and probed with hybridoma supernatant containing the mAbs diluted in blocker as follows: 1:10 -> 1:50 -> 1:100 for 4-2E2, 4-2A2, 4-3F11, and 4-5E11; 1:10 -> 1:20 -> 1:40 for 1-2B11 and 1-8H4. Probing was performed at 4°C overnight. For mutant surface exposure and folding analysis, dried and blocked blots were probed with the following concentrations: 4-2A2 (IgG1; 1:25 in blocker), 4-2E2 (IgG2a; 1:200), 4-3F11 (IgG1; 1:10), 4-5E11 (IgG1; 1:10), 1-2B11 (IgG1; 1:10), 1-8H4 (IgG2b; 1:5) also at 4°C overnight. Following primary probes, blots were washed three times with TBST and then probed for 1 hour at room temperature with either AP or HRP-labeled anti-mouse-IgG secondaries
(1:3000 in blocker). Blots were washed again and signal was developed with CN/DAB substrate for HRP, and NBT/BCIP for AP. For S100A7 blocking assays by hybrid antisera or lavages, blots were loaded, dried, and blocked in 5% BSA as described, then returned to the dot blotter. Serum or lavage samples were diluted to 3% final volume in blocker and added to the wells for 1 h, then siphoned off via vacuum. Blots were then probed with 0.1 μM S100A7-HRP for 1 h and developed as described.

X. ELISAs

For conformational testing with the mAbs, 100 μL of His-tagged TdfJ (WT and mutants) or TdfH was seeded into the wells of a Ni-NTA-coated ELISA plate at 1 μM. Proteins were allowed to bind for 2 h at room temperature or overnight at 4°C. Protein was siphoned off by vacuum and wells were blocked with 200 μL of 5% BSA (w/v) in TBST for 1 h at room temperature. After blocking, wells were probed with 100 μL of mAb (4-2A2, 4-2E2, 4-3F11, 4-5E11, 1-2B11, 1-8H4) supernatant diluted in blocker (1:100 for all) for 2 h at room temperature. Liquid was again siphoned off and wells were washed three times with 200 μL TBST for ten minutes each. HRP-labeled anti-mouse IgG was diluted in blocker (1:3000) and 100 μL was added to the wells for secondary probing for 1 h at room temperature. After siphoning and three more washes, HRP signal was developed by addition of 100 μL TMB substrate and the reaction was neutralized by 100 μL 180 mM H₂SO₄. Coloration was quantified by reading absorbance at 450 nm using a Cytation5 plate reader (BioTek). For S100A7 binding assays, 100 μL of His-tagged TdfJ (WT and mutants) were seeded into the Ni-NTA plates at 1 μM and allowed to bind for 1 h at room temperature. Wells were blocked as described above, then 100 μL of 1 nM S100A7-HRP in blocker was added to the wells and
probed for 1 h. Liquid was again siphoned away and wells were washed three times with 200 μL TBST. HRP signal was developed as described above, and all A$_{450}$ values were standardized as a percentage of wild-type binding. For detection of anti-TdfJ IgG and IgA in hybrid antisera and lavages, gonococcal cells were grown on GCB plates + 1 mM IPTG and resuspended in PBS at OD$_{600}$ = 0.2, then added to either a 384 or 96-well plate and allowed to dry. Once dried, plated were blocked with 5% BSA (w/v) PBS for 1 h, then probed with serum or lavage diluted in blocker for 2 h at room temperature. For serum, samples were diluted to 1:250, 1:500, 1:1000, and 1:2000 in 5% BSA, and lavages 1:8, 1:16, 1:32, and 1:64 in 1% BSA. After primary incubation, plates were washed three times with PBS using an EL406 liquid handling robot (BioTek), then probed with either anti-mouse-IgG or anti-mouse-IgA (both 1:5000 in blocker, both HRP conjugates) for 1 h. Following three more washes, HRP signal was detected as described above.

**XI. Surface Plasmon Resonance**

All SPR experiments were performed using an OpenSPR XT (Nicoya), and trace analysis was conducted using TraceDrawer (Ridgeview). All reagents were prepared according to the manufacturer’s instructions using de-gassed buffers. TdfJ and S100A7 were buffer exchanged into de-gassed PBS pH 8.0 prior to experiments, and all dilutions were performed using the same buffer. His-tagged streptavidin was reconstituted in de-gassed dH$_2$O prior to use. For binding experiments, NTA-coated sensor chips were first cleaned via successive injections of 10 mM HCl (150 μL/min.) and 350 mM EDTA (100 μL/min.), followed by surface activation with an injection of 40 mM NiCl$_2$ (20 μL/min.). For ligand immobilization, WT or K261P/R262P TdfJ containing a 10X N-terminal His-tag were injected
into flow channel 2 at a concentration of 89 μg/mL (20 μL/min.), and remaining NTA groups were blocked via addition of 50 μg/mL His-tagged streptavidin into both channels 1 and 2 (20 μL/min.), allowing channel 1 to serve as a reference for nonspecific interaction. S100A7 was injected (20 μL/min.) into both channels in successive steps using the following concentrations: 1, 10, 50, 100, and 500 nM. No chip regeneration step was found to be necessary. Sensorgrams were exported to TraceDrawer, where readouts for each concentration were aligned and bubble peaks were removed. For affinity analysis, sensorgrams from multiple, independent experiments were combined, aligned, and used to generate a single binding curve interval, which was then analyzed using a single site fit model and the EC50 analysis setting. In total, 13 data points were used in the WT affinity model, and 10 were used in the attempted fit of the K261P/R262P mutant.

XII. Zinc Internalization Assay

Gonococcal cultures were grown as described in CDM until exponential phase. At this point, cultures were back diluted to ½ their original density and supplemented with 1 μM Zn-loaded S100A7 and 1 mM IPTG where appropriate. Cultures were grown for 4 h before cell pellets were harvested by centrifugation (10,000 x g for 10 minutes). Pellets were washed twice with buffer containing 10 mM HEPES + 1 mM EDTA, then once more with 10 mM HEPES only. Cell pellets were frozen at -20°C before being sent to the Plasma Chemistry Laboratory at the University of Georgia Center for Applied Isotope Studies for metal analysis by inductively-coupled plasma mass spectrometry. Data are reported as micrograms of zinc per gram of cell pellet.
LITERATURE CITED


68. Prevention CfDCa. 2006. Update to CDC's sexually transmitted disease treatment guidelines, 2006: fluoroquinolones no longer recommended for treatment of gonococcal infections. Centers for Disease Control and Prevention, Atlanta, GA.

69. Prevention CfDCa. 2012. Update to CDC's sexually transmitted diseases treatment guidelines, 2010: oral cephalosporins no longer recommended treatment for gonococcal infection. Centers for Disease Control and Prevention, Atlanta, GA.


229. Korndörfer IP, Brueckner F, Skerra A. 2007. The crystal structure of the human (S100A8/S100A9)2 heterotetramer, calprotectin, illustrates how conformational


324. Liu Y, University at Buffalo M, Immunology FMSBNYUS, wynruli@buffalo.edu, Feinen B, Fda CLDBMDUS, Brandon.Feinen@fda.hhs.gov, Russell MW, University at Buffalo M, Immunology FMSBNYUS, russellm@buffalo.edu. 2011. New Concepts in Immunity to Neisseria Gonorrhoeae: Innate Responses and Suppression of Adaptive Immunity Favor the Pathogen, Not the Host. Frontiers in Microbiology 2.


411. Cash DR. 2016. DRUG AND VACCINE DEVELOPMENT FOR NEISSERIA GONORRHOEAEAVirginia Commonwealth University.


VITAE

Stavros Maurakis was born on April 12, 1992 in Danville, VA. He attended George Washington High School in Danville, where he was very active in the school’s music program and with the school’s soccer team. In 2014, he received his B.S. in Microbiology and Immunology from Virginia Tech in Blacksburg, VA. After graduation, he worked as a research and development chemist for two years before enrolling in a master’s degree program at Virginia Commonwealth University in Richmond, VA. He completed his M.S. in Microbiology and Immunology in 2019 before matriculating into the PhD program at Georgia State University in Atlanta, GA, both under the direction of Dr. Cynthia Nau Cornelissen. He finished his PhD in summer 2022 and will next begin his postdoctoral training at the National Institutes of Health in Bethesda, MD under the tutelage of Dr. Susan K. Buchanan. His publications and presentations are listed below.

Publications


Presentations

“Characterizing Hybrid TdfJ Loop Antigens for a Potential Gonococcal Vaccine”; Talk delivered at NIH Sexually Transmitted Infections Cooperative Research Centers meeting, Virtual, July 2022

“Mutation of Gonococcal TdfJ Results in Abrogation of S100A7 Binding and Utilization as a Zinc Source”; Poster presentation at American Society for Microbiology (ASM) Microbe, Washington, D.C., June 2022

"Zinc uptake systems as potential vaccine targets to protect against gonorrhea”; Talk and poster presentation for NIH Sexually Transmitted Infections Cooperative Research Centers meeting, Virtual, June 2021

"Assessing the Role of TdfJ During Gonococcal Infection"; Talk delivered at Neisseria gonorrhoeae Research Society Conference, Virtual, October 2020

“The Human Pathogen Neisseria gonorrhoeae Overcomes Host Zinc Restriction by Binding S100A7”; Poster presentation at American Society for Microbiology (ASM) Microbe, San Francisco, CA, June 2019

“Neisseria gonorrhoeae Subverts Nutritional Immunity by Co-opting Human Antimicrobial S100 Proteins”; Poster presentation at International Pathogenic Neisseria Conference, Pacific Grove, CA, September 2018