2020

The characterization of novel transgenic murine models of Neisseria gonorrhoeae infection and development of a natural outer membrane vesicle anti-gonococcal vaccine candidate

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Dissertation

THE CHARACTERIZATION OF NOVEL TRANSGENIC MURINE MODELS OF NEISSERIA GONORRHOEAE INFECTION AND DEVELOPMENT OF A NATURAL OUTER MEMBRANE VESICLE ANTI-GONOCOCCAL VACCINE CANDIDATE

by

IAN P. FRANCIS

B.S., University of Denver, 2012

Submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

2018

Approved by

First Reader _____

Lee Wetzler, M.D.
Professor of Medicine
Professor of Microbiology

Second Reader _____

Deborah Anderson, Ph.D.
Professor of Obstetrics and Gynecology
Professor of Microbiology

“We are just an advanced breed of monkeys on a minor planet of a very average star. But we can understand the universe. That makes us something special.”

-Prof. Stephen Hawking

DEDICATION

For the gifted educators and mentors that identified and encouraged my fascination with the physical world.

For my family, whose support and guidance has helped me to become a better scientist, and more importantly a better person.

For Veronica, I can't imagine a better partner with whom to tackle the challenges of life.

ACKNOWLEDGMENTS

Like with any meaningful work, the science that I present here was made possible only through the contributions and support of countless individuals. First and foremost, I owe an enormous debt of gratitude to the members, past and present, of the Wetzler lab. The success of my work is thanks to the great minds and gifted scientists that helped train and mentor me. In particular, Drs. Michael Reiser and Munir Mosaheb helped me to develop my scientific mind and were wonderful resources as I began my own work. Dr. Yazdani Shaik-Dasthagirisaheb (Basha) was an invaluable mentor and partner in this project. I hope that I have helped and taught Tina and Rachel half as much as they have imparted on me. Dr. Xiuping Liu is the rock upon which the Wetzler lab exists. Mama Ping, the love and care with which you support and aid the development of young scientists is the single greatest asset of the lab. Of course, this group would never have the chance to come together without the vision and guidance of Dr. Lee Wetzler.

I am so lucky to have completed my PhD work in Dr. Wetzler's lab. I cannot imagine I would have had the same success or learned quite as much if I had worked anywhere else. Dr. Wetzler has built a lab culture that fosters high quality and impactful science while prioritizing the growth, development, and personal wellbeing of his trainees. This is partially due to the freedom granted to students to develop their own ideas. This is what makes Dr. Wetzler such an effective mentor. He discusses and explores science with his trainees, rather than dictating it to us. I am thankful to have been able to complete my scientific training under such a mentor.

In addition to the Wetzler lab, I have been lucky enough to work in close collaboration with Dr. Scott Gray-Owen of the University of Toronto and his graduate student Dr. Epshita Islam. It was an invaluable experience to work within an international collaboration and a true pleasure to do so with these remarkable scientists.

During my scientific explorations, I frequently found myself needing a new tool or skill in order to answer my scientific question. Thankfully I was lucky enough to find kind, generous and eager individuals that helped me overcome these obstacles. The lab of Dr. Deborah Anderson, particularly Dr. Joseph Politch, graciously provided access and support to their MagPix machine allowing me to run the 20-plex cytokine arrays. The preparation and running of the microarrays was performed by the BUMC Microarray and Sequencing core under the direction of Dr. Yuriy Alekseyev. I relied heavily upon Dr. Adam Gower and the Clinical & Translational Science Institute of BU, for the analysis of the microarray data. Finally, the beautiful electron micrographs that appear in this work were made possible through the support and tutelage of Don Gantz of Dr. Esther Bullitt's lab in the department of Physiology and Biophysics at Boston University School of Medicine.

I am eternally grateful for the continued support and guidance of my wonderful research advisory committee; Dr. Hans Dooms, Dr. Sanjay Ram, Dr. Robin Ingalls, and Dr. Deborah Anderson. They each brought a unique and helpful viewpoint to the discussions of my science and were unfailingly supportive and helpful in the evolution of my work. I particularly need to thank Dr. Ram who travelled from University of Massachusetts Medical Center in Worcester for each of our meetings, Dr. Anderson who

acted as my second dissertation reader, and Dr. Ingalls who was my committee chair. I owe each of them an enormous thank you and will miss our scientific conversations!

I need to recognize and thank the administrators and my peers of the MD/PhD program. I was so lucky to join such a close-knit program. I will be forever grateful to Dr. John Schwartz for recognizing my potential and inviting me to join his program. It has been quite the experience being a part of this family as it grows and changes. My student peers are wonderful sources of inspiration and support. I am particularly indebted to Stephanie Pavlovich, Aditya Mithal, Eric Brownhill, and Jarred Mondenedo; it has been an absolute pleasure working and learning with you. I want to finally thank our current administrators, Drs. Steve Borkan and Vickery Trinkaus-Randall for their enthusiastic support of my development as a physician-scientist.

Last but not least, I have enjoyed the support of the best department I could have asked for. Led by our chair Dr. Ron Corley, and director of graduate studies Dr. Greg Viglianti, our faculty have been tremendously supportive and helpful. Kathy Marinelli and Linda Parlee, were unbelievably helpful and saved me from self-inflicted administrative snafus countless times. Ditto for Thuy-Chi Ly who helped me navigate the administrative maze of the Section of ID. Finally, the students are what makes the micro department a truly special group of people. While each and every student has made a measurable impact on me and my work, I am particularly thankful for the help of Dr. Kate Sawatski, Dr. Caitlin Miller, Dr. Jess Ritter, Dr. Michelle Fleury, Alex Barron, and Matt Gagne.

THE CHARACTERIZATION OF NOVEL TRANSGENIC MURINE MODELS OF NEISSERIA GONORRHOEAE INFECTION AND DEVELOPMENT OF A NATURAL OUTER MEMBRANE VESICLE ANTI-GONOCOCCAL VACCINE CANDIDATE

IAN P. FRANCIS

Boston University School of Medicine, 2018

Ph.D. degree requirements completed in 2018

Dual M.D./Ph.D. degreed expected in 2020

Major Professor: Lee Wetzler, M.D., Professor of Medicine, Professor of Microbiology

ABSTRACT

Untreatable gonorrhea, caused by fully antimicrobial resistant *Neisseria gonorrhoeae* (GC), is a major global health threat. While a vaccine would greatly help address this crisis, development of a GC vaccine is complicated by the lack of lab models of symptomatic gonorrhea. We hypothesized that overt disease in animal models of gonorrhea is limited by the human-restriction of gonococcal virulence factors, and the impact of the reproductive hormone cycle (estrus and diestrus phases). We tested these hypotheses by examining the host response to infection in transgenic mice expressing targets of bacterial adhesion, human carcinoembryonic antigen-related cell adhesion molecules (hCEACAMs), in uterine versus vaginal infections, and in different phases of the reproductive cycle (estrus and diestrus phases). hCEACAM expression most impacted estrus phase infections, prolonging colonization in vaginal infection and inducing greater inflammation in uterine. Reproductive phase greatly influenced host

response to uterine infection as diestrus infection was more inflammatory than estrus. Phase differences in uterine infection were driven by greater activation of a chemokine-centric common anti-gonococcal response and unique induction of type 1 interferons in diestrus. These findings suggest that symptomatic uterine and vaginal GC infection can be modeled by transcervically infected wild-type diestrus mice and transgenic, vaginally-infected estrus mice, respectively.

A novel approach to GC vaccine development is also needed. Mono-antigenic vaccines have failed to produce immunity suggesting a poly-antigenic antigen, like natural outer membrane vesicles (nOMVs) may be necessary. It has been shown that any GC vaccine must lack the bacterioprotective antigen, reduction modifiable protein (RMP), and no such nOMV has been previously described. Here we report successful isolation of RMP-deficient nOMVs through sequential size and weight restrictive filtration. Vesicle morphology, proteomics, and bioactivity was characterized via various methods. nOMVs were found to be consistent in size, shape and antigenic load. As antigens, nOMVs induced high serum titers and measurable vaginal levels of antigen and GC specific IgG that recognized several nOMV immunogens supporting the vaccine potential of GC nOMVs. These findings lay the groundwork for protective studies of nOMV vaccines in novel models of active gonorrhea moving the field closer to discovering the mechanism of protective anti-gonococcal immunity.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AZM ..... Azithromycin

BUSM ..... Boston University School of Medicine

C(d) ..... Cumulative percent contribution

CCAC ..... Canadian Council on Animal Care

CDC ..... Centers for Disease Control and Prevention

CEACAMs ..... Carcinoembryonic antigen-related cell adhesion molecules

CFM ..... Cefixime

CIP ..... Ciprofloxacin

cOMV ..... Chemically extracted outer membrane vesicle

CRO ..... Ceftriaxone

Ct ..... *Chlamydia trachomatis*

d(nm) ..... Diameter in nanometers

DFC ..... Differential fold change

DLS ..... Dynamic Light Scattering

dOMV ..... Detergent extracted outer membrane vesicle

Dox ..... Doxycycline

EM ..... Electron Micrograph

FDR ..... False Discovery Rate

FGT ..... Female genital tract

G(d) ..... Relative percent contribution of the size range

GC ..... *Neisseria gonorrhoeae* or gonococcus

GO ..... Gene Ontology

GSEA ..... Gene Set Enrichment Analysis

hCEACAMs ..... Human carcinoembryonic antigen-related cell adhesion molecules

HEK ..... Human Embryonic Kidney cells

HPI ..... Hours post infection

IACUC ..... Institutional Animal Care and Use Committee

IN ..... Intranasal (immunization)

IPA ..... Ingenuity Pathway Analysis

ITAM ..... Immunoreceptor tyrosine-based activation motif

ITIM ..... Immunoreceptor tyrosine-based inhibitory motif

LASC ..... Laboratory Animal Science Center

LC-MS ..... Liquid Chromatography-Mass Spectrometry

LOS ..... Lipooligosaccharide

MIC ..... Minimum inhibitory concentration

mOMV ..... Mechanically extracted outer membrane vesicle

MSigDB ..... Molecular Signatures Database

MSM ..... Men who have sex with men

NFDM ..... Non-fat dry milk

Nm ..... *Neisseria meningitidis*

nOMV ..... Naturally released outer membrane vesicle

NUSE ..... Normalized Unscaled Standard Error

OFX ..... Ofloxacin

OMV ..... Outer Membrane Vesicle

PAGE ..... Polyacrylamide Gel Electrophoresis

PAMP ..... Pathogen associated molecular pattern

PBS ..... Phosphate buffered saline

PC ..... Principal Component

PCA ..... Principal Component Analysis

PEN ..... Penicillin

PID ..... Pelvic Inflammatory Disease

PnPP ..... *p*-nitrophenyl phosphate

PRR ..... Pattern Recognition Receptor

RLE ..... Relative Log Expression

RMA ..... Robust Multiarrray Average

Rpm ..... Rotations per minute

SC ..... Subcutaneous (immunization)

SDS ..... Sodium dodecyl sulfate

SNP ..... Single nucleotide polymorphism

SPT ..... Spectinomycin

STI ..... Sexually Transmitted Infection

SUL ..... Sulfonamides

TEM ..... Transmission Electron Microscopy

TET ..... Tetracycline

TLR ..... TOLL-like Receptors

UA.....Uranyl-Acetate

UGT .....Upper genital tract

WHO.....World Health Organization

Chapter 1: Introduction

The History of Neisseria gonorrhoeae and Clinical Spectrum of Gonorrhea

Gonorrhea, caused by *Neisseria gonorrhoeae* (GC or gonococcus), has a long history as a cause of human disease. The earliest known written description of a gonorrhea-like condition is found in an ancient Egyptian text known as *The Ebers Papyrus* that dates to at least 1500BCE (Bryan, 1930). The papyrus describes a multitude of diseases and their contemporary treatments including a purulent vulvo-vaginal inflammation that sounds strikingly similar to female gonorrhea. Perhaps the most well-known early description of a sexually transmitted infection (STI), thought to be gonorrhea, comes from *The Book of Leviticus* 15:2-12 in the *Old Testament*: "Every man who is afflicted with a chronic flow from his private parts is thereby unclean" (*The Bible*, The New American Bible). It is thought that this is referring to the characteristic purulent discharge of gonococcal urethritis (Bingham, 2014). Of course, nearly all of these early texts describe human ailments only in the vaguest terms, and since most early texts were religious in nature, diseases, particularly those relating to the genitals, were presented as signs of moral corruption and deviancy. Until the medical revolution of ancient Greece, there were few attempts to meticulously describe, name or identify the many disorders of the human body. As such, it was one of the great ancient Greek physicians, Galen, who in the second century CE coined the term "Gonorrhoea", meaning "flowing seed" in reference to the characteristic mucopurulent discharge (Bingham, 2014).

The bacterium that causes gonorrhea, *Neisseria gonorrhoeae*, was finally isolated and described in an 1879 publication from Albert Neisser (for whom the genus of

bacteria "*Neisseria*" is named) in which he reported microscopic analysis of samples from 26 adults with classic urethritis, and 7 children and 2 adults with opthalmia (Neisser, 1879). Dr. Neisser described a "micrococcus" of standard morphology which stained with methyl violet that could be found in samples from all 35 patients. In his next publication he expanded upon his morphological study, and using the terminology of Galen referred to the etiological agent of gonorrhea as a "gonococcus" (Neisser, 1882). Modern microbiology has settled upon "diplococci" or "kissing kidney beans" to describe the cellular form of the pathogen. This description evokes an appropriate image since the gonococcus, as seen in Figure 1.1, tends to appear in cellular pairs and has an elongated notched ovoid shape.

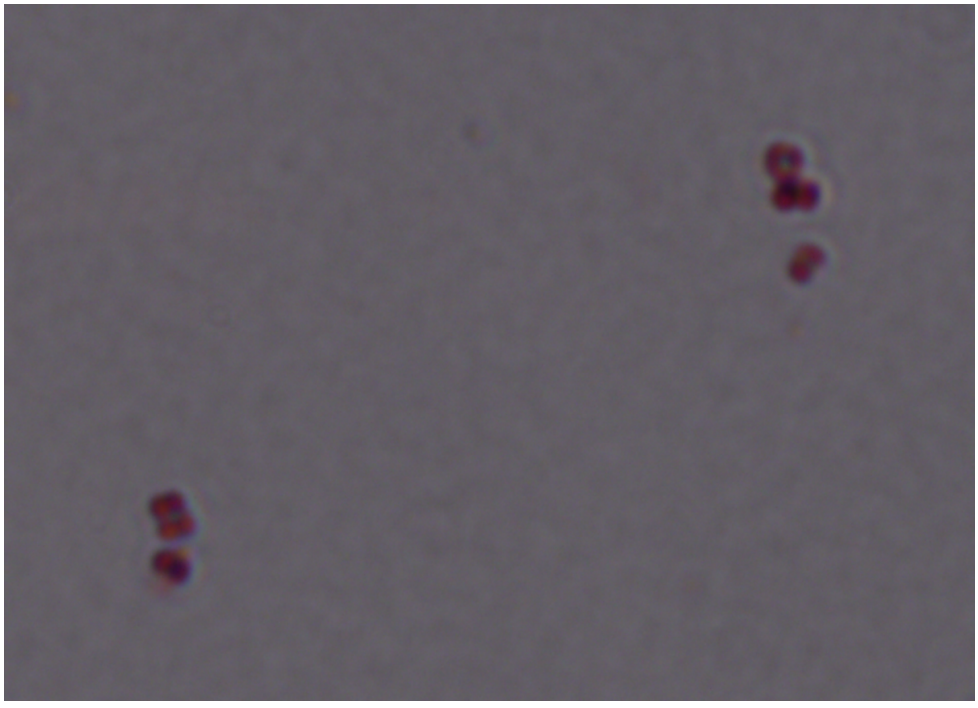


Figure 1.1: Characteristic "kissing kidney bean" morphology of the *Neisseria gonorrhoeae* diplococcus. *Neisseria gonorrhoeae* (strain MS11) from a liquid media growth heat fixed on a glass slide and visualized by Gram staining and imaged using 100x oil immersion light microscopy

Today, gonorrhea is a laboratory confirmed disease with complex clinical presentations that differ based on sex and mode of transmission. Classically, gonorrhea is thought of in terms of male and female infection in the context of transmission by heterosexual sexual contact. In this case, infection of the male genitourinary tract is thought to be symptomatic in the majority of cases (John and Donald, 1978; Harrison et al., 1979). Recent population-wide studies however, cast doubt on this figure, suggesting instead that a larger proportion (~60%) of infected males may be asymptomatic (Handsfield et al., 1974; Klouman et al., 2000). Regardless of frequency, symptomatic cases in men have a very usual presentation characteristic of the underlying condition of gonococcal urethritis. Nearly all patients report copious mucopurulent discharge from the urethral meatus commonly accompanied by dysuria (pain on urination) (Sherrard and Barlow, 1996). While some infections in men can progress in the absence of treatment to cause inflammation of the epididymis (epididymitis) this is relatively rare in isolated GC infection (Holmes et al., 1979).

In addition to the genitourinary tract, *Neisseria gonorrhoeae* can infect and cause disease in the anorectal cavity, a presentation typically found in men who have sex with men (MSM) (Kent et al., 2005; Schachter et al., 2008; Patton et al., 2014). Like in genital infection, rectal gonorrhea can be asymptomatic (Klein et al., 1977; de Vries et al., 2014). Symptomatic anorectal infections in men is termed proctitis, which presents with a constellation of symptoms that can include anorectal pain, tenesmus, mucopurulent discharge and bleeding (Klein et al., 1977). Additionally, researchers are starting to gain a greater appreciation for oropharyngeal GC colonization as a site of transmission via

oral sex (Wiesner et al., 1973), and as the likely site of horizontal transfer of antimicrobial resistance genes (Deguchi et al., 2012). Oropharyngeal colonization is found in both women (Kraus, 1979; Osborne and Grubin, 1979) and men (Patton et al., 2014) and can cause pharyngitis (Komaroff et al., 1980).

The natural course of infection in women is entirely different. While the exact proportion of symptomatic to asymptomatic cases of female gonococcal infection is a point of debate, most studies agree that the majority of female infections are asymptomatic (McCormack et al., 1977b). In both cases the typical site of bacterial colonization and growth is the cervix (Evans, 1977; Edwards et al., 2001). Symptomatic infected women most frequently have developed ether cervicitis or urethritis and present with the typical complaints associated with those conditions; mucopurulent vaginal discharge and dysuria respectively (Barlow and Phillips, 1978). In the absence of treatment, all infected women, symptomatic or not, are at risk of developing the most concerning manifestation of gonococcal infection, Pelvic Inflammatory Disease (PID).

PID is a clinical condition defined as an infection of the upper female genital tract (UGT) including the uterus, fallopian tubes, and/or ovaries (Kasper et al., 2016). Several different pathogens are capable of causing PID, however *Neisseria gonorrhoeae* and *Chlamydia trachomatis* (Ct) combine to cause the majority of cases (Soper et al., 1994). The proportional breakdown of GC versus Ct associated PID tends to reflect the underlying relative frequency of the two pathogens in a given population (2014; 2017). Regardless of the underlying microbiology etiology, PID presents clinically in a similar way. PID is associated with a large spectrum of pathology, including subclinical damage

which is only discovered years later (Molander et al., 2003; Hebb et al., 2004). Most women with PID however, are symptomatic. The most common complaints at presentation are abdominal pain and tenderness (Ross et al., 2014; Workowski et al., 2015). The characteristic physical findings are cervical, adnexal and uterine tenderness upon bimanual examination (Peipert et al., 2001; Wiesenfeld et al., 2005). Tubal infection can lead to the development of tubo-ovarian abscesses, which carry the risk of rupture and subsequent life-threatening peritonitis (De Temmerman et al., 2003; Powers et al., 2007). PID can even cause extra-genital disease. -FitzHugh-Curtis syndrome is a perihepatitis caused when the infection and subsequent inflammatory response of PID gains access to the peritoneal cavity. This results in inflammation of the hepatic capsule and can lead to the formation of peritoneal-adhesions and chronic pain (You et al., 2012). PID's most tangible and lasting effect is its impact on fertility through inflammatory changes to the fallopian tubes (Kaproth-Joslin and Dogra, 2013). A recent study suggested that PID carries an 18% chance of resulting in infertility (Haggerty et al., 2010). These serious complications of PID are made more concerning by the large annual incidence of gonorrhea.

The most recent data from the World Health Organization (WHO) estimates at least 106 million cases of gonorrhea occur in the world each year (Rowley et al., 2012) and the Centers for Disease Control and Prevention (CDC) places the US figure at approximately 416,000 cases (2017). Due to the confounding effect of asymptomatic infection, actual numbers could be as much as three-times higher (Satterwhite et al., 2013). Additionally, there has been a global increase in STI rates in recent years (Fenton

and Lowndes, 2004; 2017). This means that, although the most serious sequelae associated with infection are rare among the infected population, their incidence is significant. This consideration becomes even more important as *Neisseria gonorrhoeae* infections are becoming more common and much more difficult to treat.

Antibiotic resistance in Neisseria gonorrhoeae

Neisseria gonorrhoeae has a history of antimicrobial resistance that is nearly as old as the modern antibiotic era. The first anti-gonococcal antimicrobial, sulfanilamide, was discovered in the early 1900s (Oriel, 1994; Lewis, 2010). This drug was followed by several other derivatives all belonging to the Sulfonamide family; however nearly all GC isolates were resistant to every member of the drug family by the late 1940s (Dunlop, 1949; Kampmeier, 1983). Penicillin, after its 1928 discovery by Alexander Fleming, started to replace sulfonamides in the early 1940s and was initially extremely efficacious against gonococcal infection (Mahoney et al., 1943; Van Slyke et al., 1943). Despite steadily increasing resistance, as measured by increasing minimum inhibitory concentration (MIC) of penicillin (Franks, 1946; Amies, 1967), the drug was effective at increasing doses until the 1960s (Reyn et al., 1958; Martin et al., 1970; Jaffe et al., 1976; Lewis, 2010; Unemo and Shafer, 2011) when treatment failures with penicillin started to occur (Willcox, 1970; Genco and Wetzler, 2010; Unemo and Shafer, 2011). Shortly thereafter plasmid based β-lactamase resistance became widespread (Ashford et al., 1976; Percival et al., 1976; Phillips, 1976), followed by chromosomal mediated resistance (Faruki et al., 1985; Faruki and Sparling, 1986) thereby rendering penicillin ineffective as

an anti-gonococcal antimicrobial. As shown by Figure 1.2, this pattern of antibiotic introduction, brief period of efficacy, era of waning efficacy/rising MICs followed by treatment failures and widespread loss of efficacy against *Neisseria gonorrhoeae* has been repeated over and over since penicillin (Unemo and Shafer, 2014). Thankfully, as one antimicrobial lost efficacy against GC, there was a new anti-gonococcal therapeutic that could take its place. This pattern, however, seems to have reached its conclusion.

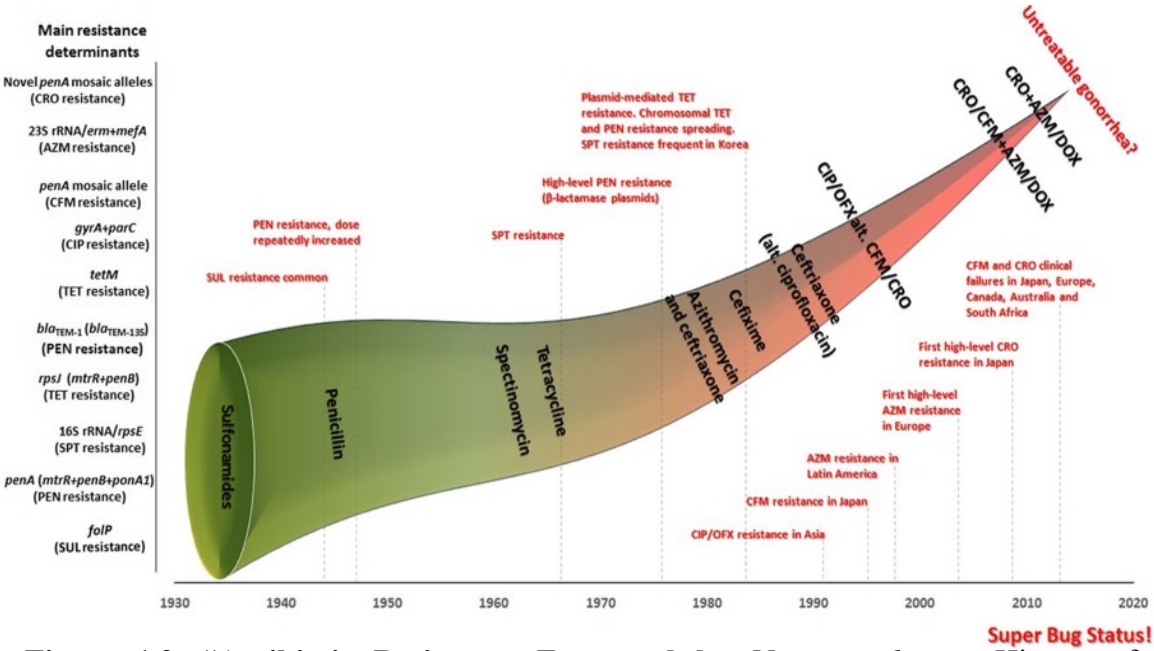


Figure 1.2: "**Antibiotic Resistance Expressed by *N. gonorrhoeae*.** History of discovered and recommended antimicrobials and evolution of resistance in Neisseria gonorrhoeae, including the emergence of genetic resistance determinants, internationally. During the pre-antimicrobial era (before the 1930s), treatment consisted of, e.g., a healthier lifestyle, copaiba, cubebs, urethral irrigations, potassium permanganate, silver compounds, mercury compounds, and hyperthermia. SUL, sulfonamides; PEN, penicillin; SPT, spectinomycin; TET, tetracycline; CIP, ciprofloxacin; OFX, ofloxacin; CFM, cefixime; CRO, ceftriaxone; AZM, azithromycin; DOX, doxycycline." Recreated here with permission, in its entirety from (Unemo and Shafer, 2014).

The current treatment guidelines for *Neisseria gonorrhoeae* infection call for dual therapy with both ceftriaxone and azithromycin (Workowski and Bolan, 2015).

Treatment failures with the macrolide, azithromycin started to appear in the late 1990s (Palmer et al., 2008; Chisholm et al., 2010; Katz et al., 2012; Unemo et al., 2014) and it hasn't been used as monotherapy since then. Even with decreased usage and the benefit of dual therapy, elevated MICs to azithromycin continue to be observed and have even increased in recent years (Prevention, 2016). The third-generation cephalosporin, ceftriaxone is the primary anti-gonococcal agent in the therapy and has suffered from worldwide progressive loss of efficacy which in many populations recently reached the level of insensitivity and resistance (Lo et al., 2008; Hess et al., 2012; Li, 2012; Unemo and Nicholas, 2012; Bala et al., 2013; Dillon et al., 2013; Kirkcaldy et al., 2013; Lahra et al., 2013; Ndowa et al., 2013). As seen with azithromycin, GC treatment failures with ceftriaxone have been documented (Deguchi et al., 2003; Ison et al., 2011; Unemo et al., 2011; Unemo et al., 2012; Allen et al., 2013; Lewis et al., 2013). It is at the intersection of these two trends toward resistance where the true public health emergency lies, as no other currently licensed antimicrobials have acceptable efficacy against GC. As ceftriaxone and azithromycin resistance spreads and co-occurs, fully drug resistant GC is the result. Such a strain was first isolated and characterized in Japan in 2011 (Ohnishi et al., 2011). Since then, several other fully drug resistant GC strains have been isolated worldwide (Unemo and Shafer, 2011; Camara et al., 2012) including a cluster of cases in Hawaii in 2017 (2016). The emergence and transmission of fully drug resistant *Neisseria gonorrhoeae* has led both the CDC (CDC, 2012) and WHO (Carmeli, 2017) to identify the development of new anti-gonococcal pharmaceuticals as "urgent" and "high" global health priorities.

The "call to arms" by these governmental and non-governmental health organizations has focused on the antibiotic resistance crisis by pushing for next generation antimicrobial development. As shown in Table 1, the gonococcus has demonstrated a remarkable ability to acquire diverse antibiotic-resistance adaptations (i.e. resistance genes, metabolic changes, mutations) through a variety of mechanisms (Unemo and Shafer, 2014). In addition, resistance to any one drug class has been achieved in several different ways. This genetic and metabolic flexibility as well as gonorrhea's repetitive history of therapeutic failure suggests that, barring a transcendent discovery, any new therapeutic will likely eventually be rendered useless. The public health crisis represented by fully drug resistant *Neisseria gonorrhoeae* is best addressed by a long-lasting, broadly-reactive gonococcal vaccine.

Antimicrobial class	Resistance determinants/mechanisms
Sulfonamides	Oversynthesis of *p*-aminobenzoic acid, which dilutes the sulfonamide. Mutations in *folP* (encoding the sulfonamide target DHPS) reduce target affinity. The *folP* mutations comprise SNPs or a mosaic *folP* gene containing sequences from commensal *Neisseria* spp.
Penicillins (e.g., penicillin G and ampicillin)	Mutations in *penA* (encoding the main lethal target PBP2). Traditionally, the mutations were the single amino acid insertion D345 in PBP2 and 4 to 8 concomitant mutations in the PBP2 carboxyl-terminal region, decreasing the PBP2 acylation rate and reducing susceptibility ~6- to 8-fold. In the last decade, many mosaic *penA* alleles with up to 70 amino acid alterations, also reducing PBP2 acylation, were described. Mutations in *mtrR*, in the promoter (mainly a single nucleotide [A] deletion in the 13-bp inverted repeat sequence) or coding sequence (commonly a G45D substitution), result in overexpression of and increased efflux from the MtrCDE efflux pump. See the text for rarer mutations resulting in increased MtrCDE efflux. *porB1b* SNPs, e.g., encoding G120K and G120D/A121D mutations in loop 3 of PorB1b, reduce influx (*penB* resistance determinants). Interestingly, the *penB* phenotype is apparent only in strains with the *mtrR* resistance determinant. A SNP in *pilQ* (encoding the pore-forming secretin PilQ of the type IV pili), i.e., E666K, reduces influx. Note that this SNP has been found only in the laboratory and is unlikely to be present in clinical isolates, because it disrupts type IV pilus formation, which is essential for pathogenesis. A SNP in *ponA* (encoding the second penicillin target, PBP1), i.e., "*ponA1* determinant" (L421P), reduces penicillin acylation of PBP1 ~2- to 4-fold. "Factor X," an unknown, nontransformable determinant, increases penicillin MICs ~3- to 6-fold. Penicillinase (TEM-1 or TEM-135)-encoding plasmids, i.e., Asian, African, Toronto, Rio, Nîmes, New Zealand, and Johannesburg plasmids, hydrolyze the cyclic amide bond of the β-lactam ring and render the penicillin inactive.
Tetracyclines (e.g., tetracycline and doxycycline)	A SNP in *rpsJ* (encoding ribosomal protein S10), i.e., V57M, reduces the affinity of tetracycline for the 30S ribosomal target. *mtrR* mutations (see above). *penB* mutations (see above). A SNP in *pilQ* (see above). TetM-encoding plasmids, i.e., American and Dutch plasmids. Evolved derivatives have been described in Uruguay and South Africa. TetM, resembling elongation factor G, binds to the 30S ribosomal subunit and blocks tetracycline target binding.
Spectinomycin	A 16S rRNA SNP, i.e., C1192U, in the spectinomycin-binding region of helix 34, reduces the affinity of the drug for the ribosomal target. Mutations in *rpsE* (encoding the 30S ribosomal protein S5), i.e., the T24P mutation and deletions of V25 and K26E, disrupt the binding of spectinomycin to the ribosomal target.
Quinolones (e.g., ciprofloxacin and ofloxacin)	*gyrA* SNPs, e.g., S91F, D95N, and D95G, in the QRDR, reduce quinolone binding to DNA gyrase. *parC* SNPs, e.g., D86N, S88P, and E91K, in the QRDR, reduce quinolone binding to topoisomerase IV. Many additional mutations in the QRDR of *gyrA* and *parC* have been described. An overexpressed NorM efflux pump also slightly enhances quinolone MICs.
Macrolides (e.g., erythromycin and azithromycin)	23S rRNA SNPs, i.e., C2611T and A2059G (in 1 to 4 alleles), result in a 23S rRNA target (peptidyltransferase loop of domain V) with a reduced affinity for the 50S ribosomal macrolide target. *mtrR* mutations (see above). *erm* genes (*ermB*, *ermC*, and *ermF*), encoding rRNA methylases that methylate nucleotides in the 23S rRNA target, block the binding of macrolides. MacAB efflux pump; its overexpression increases the MICs of macrolides. *mef*-encoded efflux pump exports macrolides out of the bacterial cell and increases the MICs of macrolides.
Cephalosporins (e.g., ceftibuten, cefpodoxime, cefixime, cefotaxime, and ceftriaxone)	Mosaic *penA* alleles encoding mosaic PBP2s with a decreased PBP2 acylation rate. These proteins have up to 70 amino acid alterations and are derived from horizontal transfer of partial *penA* genes from mainly commensal *Neisseria* spp. Mutations in mosaic PBP2s verified to contribute to resistance are A311V, I312M, V316T, V316P, T483S, A501P, A501V, N512Y, and G545S. The resistance mutations need other epistatic mutations in the mosaic *penA* allele. *penA* SNPs, i.e., A501V and A501T, in nonmosaic alleles can also enhance cephalosporin MICs. Some additional SNPs (G542S, P551S, and P551L) were statistically associated with enhanced cephalosporin MICs, but their effects remain to be proven with, e.g., site-directed *penA* mutants in isogenic backgrounds. *mtrR* mutations (see above). *penB* mutations (see above). "Factor X," an unknown, nontransformable determinant (see above).

Table 1.1: "Resistance determinants and mechanism in *Neisseria gonorrhoeae* for antimicrobials previously or currently recommended for treatment of gonorrhea." Recreated here with permission, in its entirety from (Unemo and Shafer, 2014).

Gonococcal Vaccines

The ease with which gonorrhea could be treated and the relative lack of associated severe disease, as compared to other pathogens that produced a majority of infectious

cause deaths (i.e. *S. pneumoniae*, *N. meningitidis*, poliovirus), created a lack of urgency surrounding the development of a gonococcal vaccine until recently. Additionally, the complete lack of an immunological "blueprint" for protective immunity has left the field without clarity in correlates of protection or consensus on preferred antigenic targets leaving the field with little direction when designing a candidate vaccine. As basic microbiologists have made strides in characterizing the gonococcus, their findings have inspired intermittent gonococcal vaccine studies that, while ultimately unsuccessful, provide guidance for this new wave of investigation. Based on observations of its essential role in pathogenesis (Heckels et al., 1989) and *in vitro* ability to block gonococcal binding (Meyer and van Putten, 1989), early vaccine studies focused on the gonococcal pilus as the primary antigen. While small cohort studies showed desirable *in vitro* characteristics like strong heterologous and homologous antigenic binding and interference with bacterial adhesion to epithelial cells (McChesney et al., 1982), when the pili vaccine was tried in a large cohort human study it was unable to establish significant protection against infection (Boslego et al., 1991). Subsequent microbiological studies of the gonococcus determined that like many gonococcal antigens, the pilus is subject to significant phase and antigenic variation (Seifert et al., 1994), which likely contributed to the failure of the vaccine (Meyer and van Putten, 1989). Through this trial we have been able to determine that the impact of phase variation and antigen variation is substantial. This suggests that a successful anti-gonococcal vaccine will likely need to overcome these challenges either by using an expression stable antigen or by placing overwhelming pressure on the organism through a large poly-antigenic response.

Additional lessons can be derived from a vaccine trial focused on the major gonococcal outer membrane protein, a bacterial porin protein called PorB (Blake and Gotschlich, 1982). Using a vaccine containing an 85% pure preparation of gonococcal porin, researchers evaluated the ability of the vaccine to prevent experimentally induced urethritis in men (Tramont, 1989). The vaccine showed no protective efficacy and the study was initially labeled a complete failure and was only published as part of a review. Importantly, upon subsequent analysis of immune sera it was determined that the lack of bactericidal activity and anti-PorB antibodies could be explained by an interfering antigen that had contaminated the porin preparation. When the gonococcal protein RMP is introduced as an antigen, even as a minor component, it becomes the immunodominant immunogen and induces antibodies that disrupt the bactericidal activity of normally bactericidal antibodies recognizing other gonococcal antigens (Gulati et al., 1991; Rice et al., 1994). The effect of the bacterioprotective anti-RMP response is so great that it increases a person's susceptibility to GC infection (Plummer et al., 1993). Based on these studies it is clear that any gonococcal vaccine must be entirely pure of RMP contamination. To this end, *rmp* deletion mutant strains of GC have been derived (Wetzler et al., 1989) and serve as the platform for antigen purification for use in vaccines (Wetzler et al., 1988; Blake et al., 1989; Wetzler et al., 1992b).

While previous gonococcal vaccine trials have failed to define protective correlates or discrete goals for the next generation of vaccines, they have been instrumental in informing the theory behind our next steps of development. The most valuable lessons have been regarding the type(s) of antigen(s) that are needed to evade

the gonococcus' characteristics that make it difficult to develop protective immunity against. Some GC studies, as well as the recently licensed group-B meningococcal vaccine, suggest that these challenges may be overcome by the use of outer membrane vesicles as complex antigens.

Outer Membrane Vesicles

Neisseria gonorrhoeae belongs to the family of bacteria known as Gram-negatives, as demonstrated by its vivid red staining in Figure 1.1. Gram-negative bacteria share, by definition, a unique cellular envelope structure. The envelope is composed of three distinct layers; an inner lipid-bilayer, a "periplasmic" space populated principally by a thin peptidylglycan wall, and an outer lipid-bilayer (Bos et al., 2007) It is the safranin staining of this outer lipid bilayer that gives Gram-negatives their characteristic red coloration following Gram staining.

As a Gram-negative bacterium, *Neisseria gonorrhoeae* undergoes a "blebbing" of the outer lipid bilayer to produce what are called outer membrane vesicles (OMVs) (Dorward and Judd, 1988). While there have been recent discoveries that suggest membrane blebbing may not be a uniquely Gram-negative phenomenon (Rivera et al., 2010), we will focus on Gram-negative vesicles since they are most relevant to the bacterium of interest, *Neisseria gonorrhoeae*. OMVs were first described in a 1967 study of *Vibrio cholera* growth and were thought to be an artifact of laboratory culture (Chatterjee and Das, 1967). Since then, structures similar to these have been observed and characterized from a large array of Gram-negative bacteria, both commensals and

pathogens (Kulp and Kuehn, 2010). The frequency and repetition of these observations, as well as some common characteristics between vesicles of different bacteria has led to acceptance of the manufacture of OMVs as an active and conserved process in Gram-negative bacteria (Kulp and Kuehn, 2010).

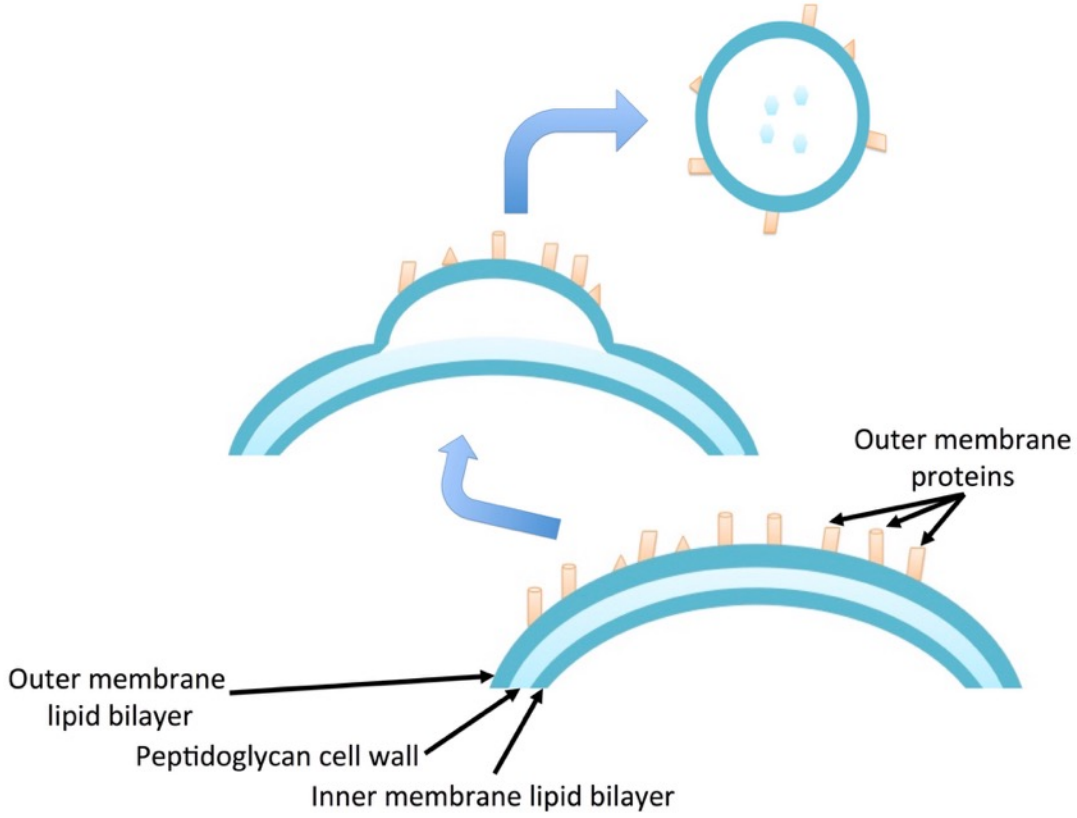


Figure 1.3: **A graphical representation of Gram-negative bacterial envelope structure and outer membrane vesicle genesis**. Gram-negative associated outer membrane vesicles (OMVs) are released naturally in response to an unidentified signal. As represented here, the vesicles carry a subset of outer membrane associated proteins as well as some periplasmic debris within their single-lipid-bilayer-bound lumen.

The body of OMV literature remains thin, particularly for any one pathogen, however a general understanding of the role and function of vesicles can be appreciated by examining all findings together. It has been shown that OMVs are single lipid bilayer

spheres which carry an array of outer membrane derived lipids, proteins, and LPS (Pettit and Judd, 1992; Vanaja et al., 2016), periplasmic peptidylglycan and proteins (Kaparakis et al., 2010), and finally cytoplasmic proteins and nucleic acids (Lindmark et al., 2009; Koeppen et al., 2016) (Figure 1.3). The exact composition of the vesicles, particularly proteomic composition, provides some of the most compelling evidence for the purposeful production of the vesicles. Several studies have examined the proteome of vesicles and compared it to both the bacterial proteome and outer-membrane proteome at the time of blebbing (Olofsson et al., 2010; McMahon et al., 2012; Zielke et al., 2014). In each of these studies, including Zielke and colleagues' examination of GC OMVs, it was shown that OMVs contain a predictable subset of proteins in different relative concentrations from the source outer membrane and bacterium. This suggests active sorting of proteins to curate the OMV proteome, likely to facilitate their function.

Selective enrichment of certain proteins in OMVs is illustrated by shiga-toxin producing *E. coli* which has been shown to increase secretion of shiga-toxin through both direct secretion into the extracellular space and release of toxin containing OMV (Yokoyama et al., 2000). This use of OMVs as a vector to deliver toxins or other virulence factors is one of the suspected functions of the vesicles. For several toxin-producing bacteria, including shiga-toxin expressing enterohemorrhagic *E. coli*, it has been shown that toxin activity is greater when delivered in secreted OMVs than as soluble protein (Yokoyama et al., 2000; Wai et al., 2003; Kuehn and Kesty, 2005). Vesicles also seem to deliver and increase activity of virulence factors. OMVs derived from *M. catarrhalis* are known to be enriched with bacterial adhesions that facilitate the

binding and retention of bacterial colonies to host tissues (MacDonald and Kuehn, 2012). Vesicles can also be used by their parent bacterium to manipulate the host organism. The delivery of protein and non-protein pathogen associated molecular patterns (PAMPs) has been shown to induce high levels of inflammation and a non-specific host response, which may undermine a specific adaptive immune response (Ellis and Kuehn, 2010; Kaparakis et al., 2010; Winter et al., 2014). In addition to their anti-host function, OMVs also can also work in a defensive role as decoys. This hypothesized function is one that has proven to be difficult to test; however, it is known that both innate and adaptive elements of the host immune system have been shown to interact and respond to vesicles as if they were bacterial cells, thereby interfering with their anti-bacterial functions (Yanez-Mo et al., 2015). Finally, vesicles are thought to play a critical role in the formation and maintenance of large bacterial colonies through the transfer of metabolites, waste, and signal molecules (Mashburn and Whiteley, 2005). A possible role for OMVs in biofilms and dense cultures seems likely since blebbing occurs most frequently in high density growth conditions (Klimentova and Stulik, 2015).

Even though the "when" of OMV formation has been extensively described, the "how" has yet to be determined. Since the process of blebbing appears to be conserved among Gram-negatives, it has been thought that there would be a common mechanism of OMV production. So far, the few productive studies of this process instead suggest diverse and unique processes for each step in OMV formation from stimulating signal to release (Pathirana and Kaparakis-Liaskos, 2016). The most compelling evidence for the stimulating signal for OMV production was described for *P. aeruginosa*, where curvature

of the outer membrane and eventual release of OMVs was induced by integration of the quorum sensing molecule, PQS, into the outer membrane (Mashburn and Whiteley, 2005; Lee et al., 2016). Unfortunately, the same studies showed that OMVs can form in the absence of PQS suggesting that there are likely several pathways that result in OMV production. Additionally, no other quorum sensing molecule has been shown to have the same direct effect on membrane blebbing suggesting this pseudomonal mechanism may be unique.

One of the challenges that have slowed the investigation of OMVs, and extracellular vesicles in general, is the lack of strict defining characteristics (Yanez-Mo et al., 2015). Researchers define OMVs by the rough criteria of size and morphology (Kulp and Kuehn, 2010). Even now, after the advent of molecular biology, these descriptive characteristics rather than protein or molecular markers remain the defining characteristics of OMVs. The study of OMVs is only further complicated by the extreme difficulty associated with the pure isolation of the particles, especially in quantities large enough for experimentation (Yanez-Mo et al., 2015).

Since no definitive definition of an OMV exists, methods of "OMV" isolation were developed based on their ability to produce intact structures bound by lipid-bilayer(s) with a rough size between 20 and 300 nm containing some outer membrane proteins from the source bacterium. Based on this definition, the target of most of the developed methods of isolation was the bacterial lipid-bilayer. The most widely used method that was developed used a bile acid, deoxycholic acid, to chemically disrupt the lipid bilayers of the bacteria resulting in detergent derived vesicles (dOMVs) (Claassen et

al., 1996). Several others methods were also developed like the mechanical shearing of OMVs from cells by passing them through a narrow-gauge needle (mOMVs) (Zhu et al., 2005), or using chelators to disrupt the association of the outer bilayer with the underlying cell wall thereby promoting vesicle (cOMVs) release (Keiser et al., 2010). In addition to these methods, some researchers continued to isolate and characterize vesicles that were naturally released by bacteria (nOMVs) (Post et al., 2005).

As the field moved forward with the characterization of their "OMVs", conflicting observations started to become commonplace (Yanez-Mo et al., 2015). Following the detailed molecular characterization and comparison of cOMVs, dOMVs, mOMVs, and nOMVs it became clear that these structures were very different from one another in composition (Post et al., 2005; Lappann et al., 2013; van de Waterbeemd et al., 2013). These molecular studies allowed for a greater understanding of how these different classes of OMVs differ. It was shown that dOMVs contained much greater quantities of cytoplasmic compounds than the other vesicle forms (van de Waterbeemd et al., 2013). In contrast, cOMVs and nOMVs are both enriched with outer membrane lipoproteins but contain different cohorts in ratios distinct from each other (van de Waterbeemd et al., 2013). It has also been shown, though not as completely, that there is significant batch variability with all OMVs, especially when isolated through secondary mechanisms (cOMVs, mOMVs, dOMVs) (van de Waterbeemd et al., 2010; van der Pol et al., 2015). These multiple sources of variability in molecular composition likely are the cause of the conflicting results in early *in vitro* experiments of OMV function (Yanez-Mo et al., 2015). Like with any pharmaceutical, consistency in composition and effect are

characteristics required of a vaccine antigen. Variations in formulation can result in batch dependent differences in protection efficacy, or even unpredictable adverse events. The body of OMV literature suggests that nOMVs demonstrate the most consistency in composition, provided they are prepared under similar and reproducible conditions (Post et al., 2005; Kulp and Kuehn, 2010; van de Waterbeemd et al., 2010).

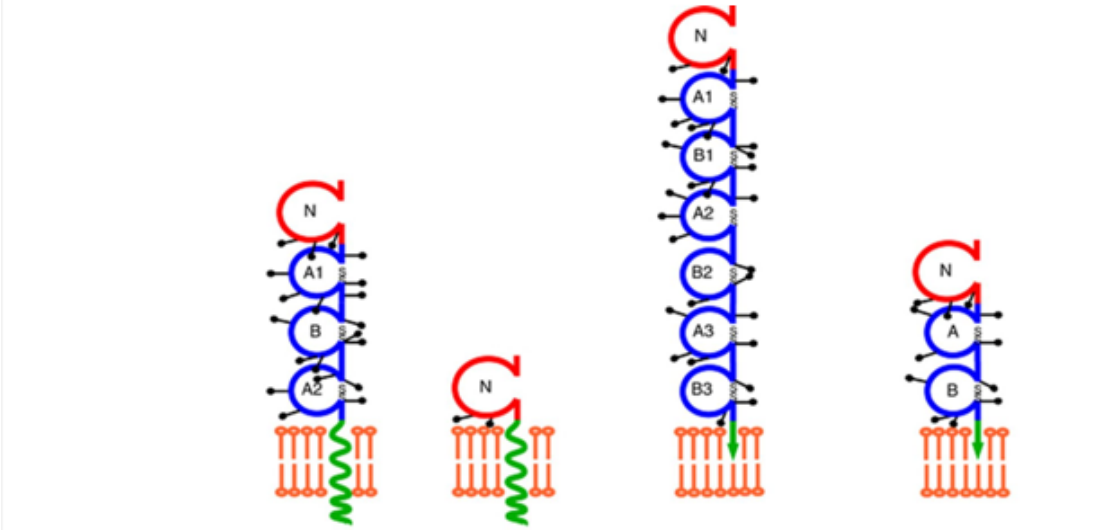
While there is variability in described OMV formation, composition, and function, their potential as complex vaccine antigens are clear. The target of a neutralizing vaccine, like one that would prevent gonorrhea by blocking initial adhesion to the mucosal surface, is most frequently an externally-exposed membrane structure. In OMVs there are a broad array of outer membrane structures that are presented in their native form since they are derived from the outer membrane of the actual pathogen. This should ensure that the faces of antigens that the immune system initially comes into contact with should be externally exposed. This will hopefully ensure, following booster exposure, that the trained immunological memory is focused on the externally exposed epitopes of membrane bound structures. This will select for the type of antigen that typically mediates binding. As demonstrated by previous studies, the induction of a unidimensional immune memory does not result in protection against GC. OMVs, in addition to likely inducing some degree of neutralizing immunity, will induce a remarkably broad immune response due to their molecular diversity. It is possible that this broad immunological pressure could overwhelm the gonococcus and successfully induce protective immunity (van der Pol et al., 2015).

Host restriction of gonococcal binding factors

As previously discussed, the gonococcus has likely existed as a human pathogen for over 3000 years (Bryan, 1930). During this microbiological eternity, the bacterium has adapted itself to human physiology, carving out a stable lifecycle of transmission, colonization/disease, and further transmission within human populations. The result of these single-species specific adaptations is that GC is unable to naturally colonize, let alone cause disease, in any organism besides humans (Johnson et al., 1977). This species restriction is often attributed to the inability of key gonococcal virulence factors to interact and carry out their function with any host target besides the human form. As demonstrated by the increased risk for neisserial infections in individuals with complement deficiencies (Figueroa and Densen, 1991), complement based bacterial killing is a crucial host defense against pathogenic *Neisseria* species. To undermine this protective host mechanism, the gonococcus has adapted to be able to bind both human Factor H (Ram et al., 1998a; Ram et al., 1998b; Ngampasutadol et al., 2008) and C4b-binding protein (Ngampasutadol et al., 2005), inhibitors of the alternative and classic/lectin complement activation pathways respectively. When unable to exploit these natural complement inhibitors, GC loses a major immune-evasion mechanism and decreases its infectious fitness. It has also been shown that the ability of GC to acquire iron, a critical metabolic co-factor, is diminished in the context of non-human physiology due to its inability to interact with non-human forms of lactoferrin and transferrin (Lee and Schryvers, 1988). However, in the context of mucosal colonization and localized

infection, we believe it is the host restriction of bacterial adhesion targets, in particular the opacity (Opa) proteins, that most impacts the ability to model gonorrhea.

Neisseria gonorrhoeae species can carry up to 11 different isoforms of the Opa protein (Muralidharan et al., 1987). As a class, these proteins target and bind to select members of the human carcinoembryonic antigen-related cell adhesion molecule (hCEACAM) family (Bos et al., 1997) without any cross recognition of non-human CEACAMs (Voges et al., 2010). These glycoproteins belong to the cell-adhesion molecule immunoglobulin superfamily. As such, each member contains at least one characteristic immunoglobulin-like domain (Vaughn and Bjorkman, 1996) which serves as the scaffolding to build a receptor structure. These domains are dense folds formed by two β-sheets. Within the superfamily of immunoglobulin cell adhesion molecules, these immunoglobulin folds have developed to recognize and bind other self-molecules typically from the same class (Crossin and Krushel, 2000). Through these interactions, CEACAMs mediate direct cell-to-cell recognition and are active in a very broad range of normal cellular processes. The relevant functions of the hCEACAMs targeted by *Neisseria gonorrhoeae* (CEACAMs 1, 3, 5, and 6) are related to their epithelial and/or leukocyte expression (Sadarangani et al., 2011). As shown in Figure 1.4 these molecules share the basic structural component of the Ig domain; however they differ in size, tissue distribution and signaling capability.



Current protein name	CEACAM1	CEACAM3	CEA	CEACAM6
Old protein name(s)	BGP1*, TM-CEA, CD66a	CGM1†, CD66d	CD66e	NCA, NCA-50/90‡, CD66c
Gene name	*CEACAM1*	*CEACAM3*	*CEACAM5*	*CEACAM6*
Splice variants	12	3	1	1
Tissue distribution	Epithelia, neutrophils, lymphocytes, endothelia	Neutrophils	Normal epithelia, high levels in some carcinomas	Epithelia, neutrophils, monocytes

From: Opa proteins and CEACAMs: pathways of immune engagement for pathogenic Neisseria
FEMS Microbiol Rev | © 2011 Federation of European Microbiological Societies.

Figure 1.4: "Opa protein-binding receptors of the human CEACAM family. CEACAM proteins consist of a 108-amino acid N-terminal domain homologous to the immunoglobulin-variable domain (shown in red), and between zero and six domains homologous to the immunoglobulin-constant domain of the C2 set (shown in blue) (Williams and Barclay, 1988). The IgC2 domains may either be of type A (93 amino acids) or type B (85 amino acids). There are two types of membrane anchorage observed among the CEA subgroup of CEACAM proteins. CEACAM1 and CEACAM3 contain a hydrophobic transmembrane domain, followed by a cytoplasmic domain (〰). CEA (also known as CEACAM5) and CEACAM6 are attached to the cell surface via a glycosylphosphatidylinositol moiety (➡) (Hammarstrom, 1999). represent the potential glycosylation sites (Yamashita et al., 1987; Yamashita et al., 1989). CEACAM1 and CEACAM3 occur in different isoforms, derived by alternative mRNA splicing. The most important differences between these splice variants seem to be the cytoplasmic domain sequence, which determines the presence or absence of immunoreceptor tyrosine-based activation (CEACAM3) or inhibition (CEACAM1) motifs. In all forms, the N-domain is retained (Hammarstrom, 1999)." Recreated here, with permission in its entirety from (Sadarangani et al., 2011)

Based on *in vitro* characterization (Muenzner et al., 2000; Voges et al., 2010) and *in vivo* (Islam et al., 2018) studies of tissue expression distribution, it's thought that

hCEACAMs 1 and 5 mediate gonococcal binding to the upper and lower human female genital tract (FGT) epithelium respectively. Interestingly, hCEACAM1's role in GC pathogenesis may not be entirely passive. hCEACAM 1 can, depending on splice variant, have a cytoplasmic tail with an immunoreceptor tyrosine-based inhibitory motif (ITIM) which, upon activation, induces changes that counter the natural exfoliation of epithelial cells (Muenzner et al., 2005). In contrast, the binding target for GC in the lower FGT, hCEACAM5, has no signaling capability and is a passive component of pathogenesis serving only as an anchor for adhesion (Kuespert et al., 2006). The function of hCEACAM6 is not entirely clear. It is known to be expressed both on epithelial cells and leukocytes (neutrophils and monocytes specifically) and can be bound by neisserial Opas, but what role, either host detrimental or protective, it plays in GC infection is yet to be appreciated (Bos et al., 1997).

The remaining GC-relevant hCEACAM, hCEACAM3, has a unique structure and function. Like hCEACAM1 it contains a signaling cytoplasmic tail. In this case however, the cytoplasmic tail has an immunoreceptor tyrosine-based activation motif (ITAM) that becomes phosphorylated when the receptor binds the bacterial structures that are its ligand (Hauck et al., 1998). Activation mediates the rapid engulfment of the bacterial cell by the hCEACAM3 expressing granulocyte. Interestingly, activation of hCEACAM3's ITAM is also associated with activation of the GTPase Rac, which is central to the activation of oxidative burst (a key anti-bacterial function of the innate immune system) (Williams et al., 2000). These immune functions along with the failure to identify a natural human ligand for hCEACAM3 has led to it being labeled a "decoy receptor"

designed to take advantage of hCEACAM-binding pathogens (Kuespert et al., 2006). It is easy to appreciate how the absence of these various Opa-hCEACAMs interactions resulting in poor adhesion and an inability to induce epithelial retention could prevent the gonococcus from establishing colonization or infection in a non-human genital tract.

Animal models of *Neisseria gonorrhoeae* infection

Development of efficacious pharmaceuticals, antimicrobials or vaccines, is often dependent upon the laboratory models of disease. Over the centuries of co-existence, *Neisseria gonorrhoeae* has become so adapted to human physiology, that it is incapable of natural infection or even colonization in any other organism (Lee and Schryvers, 1988; Gray-Owen and Schryvers, 1993; Voges et al., 2010). This has made the *in vivo* modeling of gonorrhea extremely difficult.

Early studies of infection and disease utilized human volunteers or chimpanzees; however, modern ethical standards, prohibitive cost, and experimental limitations have rendered them non-viable for modern research (Kraus et al., 1975; Arko, 1989; Gray-Owen and Schryvers, 1993; Ramsey et al., 1994). The current standard for *in vivo* *Neisseria gonorrhoeae* experiments was introduced in 1999. This model, from here on referred to as the "estrus model", is a female wild-type mouse arrested in the estrus phase of the reproductive hormone cycle through continuous β-estradiol treatment and with an antibiotic suppressed microbiome (Jerse, 1999; Jerse et al., 2011). Under these conditions, the mouse becomes permissive to vaginal colonization by *Neisseria gonorrhoeae*. The continuation of gonococcal colonization however is dependent upon

the continuation of the microbiome suppression and estrus arrest. Discontinuing either treatment leads to rapid clearance of the gonococcus (Jerse et al., 2011).

In addition to being reliant upon a very restrictive biological state, the estrus model recapitulates only a portion of human gonorrhea's natural history, specifically asymptomatic or subclinical colonization. Upon vaginal inoculation, the estrus model displays significant, but relatively mild, induction of pro-inflammatory cytokines and influx of neutrophils to the site of infection (Jerse, 1999; Song et al., 2008; Jerse et al., 2011). While hard to quantify in mice, there seems to be little to no evidence of the hallmarks of active gonorrhea in humans (i.e. dysuria, purulent discharge), instead the estrus model seems to most closely recreate asymptomatic colonization. Unfortunately, the estrus model does not appear to allow the infection to ascend in the female genital tract and cause a PID like condition, as is seen in human colonization or infection. The model does, however, faithfully recreate the lack of adaptive immune engagement and education that is seen in humans. The mice can be infected without inducing a significant anti-GC adaptive response and no secondary adaptive system response is observed upon reinfection (Song et al., 2008). The estrus model has proven to be a powerful tool in microbiological and basic immunological studies of GC infection; however, with the critical need for next generation anti-gonococcal pharmaceuticals, it will become important to review and improve upon the model to increase the chances of identification of candidate products in the lab and successful transfer to humans. Early steps towards this goal have already been taken thanks to advances in genetic engineering. Transgenic science has progressed so that transgenes can be inserted with greater precision allowing

for the production of healthier poly-transgenic animals. Using these advances, groups have produced hCEACAM transgenic mice that could bring the biology of modeled GC infection closer to that of human infection (Chan and Stanners, 2004; Gu et al., 2010). Importantly, the goal is to produce products that prevent all manifestations of human GC infection. To that point, it will be important to have the ability to test candidate products in systems that reflect not just colonization, but symptomatic gonorrhea as well as UGT infection/PID.

The Impact of the Female Reproductive Cycle on Gonorrhea

It is possible that the key to modeling the different processes of gonorrhea may lie with the natural reproductive hormone cycle. The female genital tract undergoes significant physiological and structural changes during the ~28-day menstrual cycle (Verma, 1983; Dockery et al., 1998; Rosario et al., 2003). Some of these changes, like thinning of the cervical mucus or retrograde flow of blood during menses, can provide an avenue of access for gonococci to the normally sterile upper FGT. Epidemiological studies had in fact shown an impact of the menstrual cycle on gonorrhea. Symptomatic PID patients typically present within 10 days of the first day of menses (McCormack et al., 1977a). Progesterone levels were found to be higher in asymptomatic women from China (Wu et al., 2011). From these studies, as well as the *in vitro* characterizations of sex hormones' impact on immune function, it is clear that the complex biology of the female menstrual cycle could significantly impact the course of gonococcal infection (Braude et al., 1978).

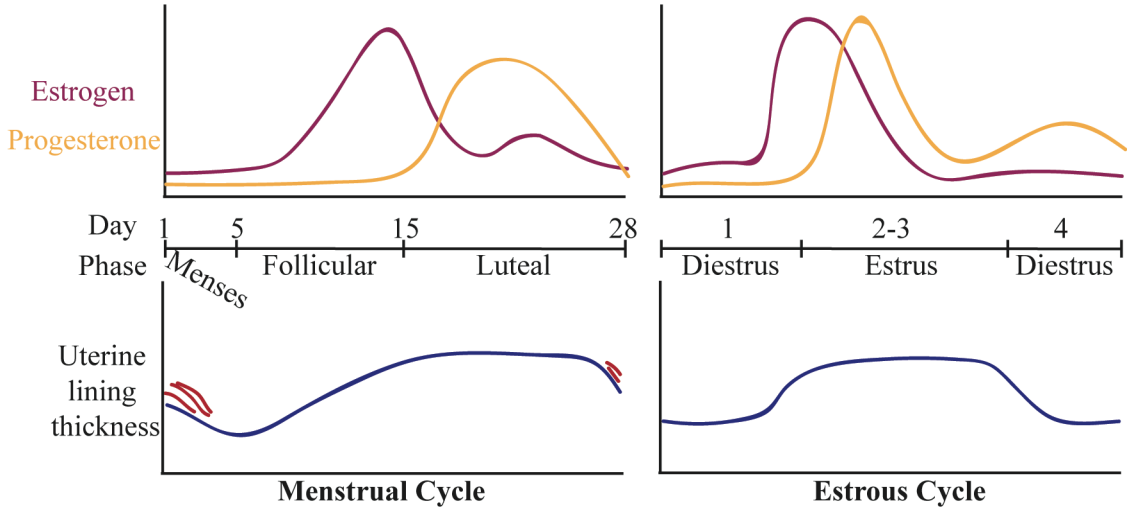


Figure 1.5: **A comparison of sex hormone levels and uterine tissue structure during the human menstrual cycle and murine estrous cycle.** Estrogen (burgundy line) and progesterone (mustard line) levels during the human menstrual cycle and murine estrous cycle are indicated in the upper left and right graphs respectively. The typical thickness of the uterine lining at various points of the menstrual or estrous cycle is shown in the bottom two graphs. The blue line represents thickness with the red marks indicating the process of menstruation. Figure based on (Staley and Scharfman, 2005; Hawkins and Matzuk, 2008)

The dependence of the estrus model on exogenous β-estradiol shows that the murine equivalent to the menstrual cycle, the estrous cycle, also has profound effects on the gonococcal infection (Jerse, 1999). The estrous and menstrual cycles are very different; however, both are driven by the two major female sex hormones, estrogen and progesterone. The greatest difference between the two processes is their duration. The estrous cycle runs approximately 4 days from start to finish during which there are two general phases; estrus and diestrus phase (Figure 1.5). While the general pattern of reproductive hormone surges is similar between the estrous and menstrual cycle, the shorter duration of the estrous cycle results in less separation between the estrogen and progesterone surge (Figure 1.5).

These hormone surges have a significant impact on the structure of the uterine lining. The estrus phase is initiated by an estradiol surge, corresponds roughly with ovulation and induces a structural state characterized by a thinned cervical mucus and a thickened uterine lining (Corbeil et al., 1985). Diestrus on the other hand is a period of deconstruction and subsequent reconstruction of the uterine lining resulting from sustained elevated progesterone levels (Staley and Scharfman, 2005). The reproductive cycle also affects the vaginal lining. One early ultrastructural study described these changes and provided potential rationale for the estrus dependence of gonococcal colonization. Corbeil and colleagues (1985) observed that there was a relative loss of columnar cells, which mediate bacterial binding, in the vagina during the diestrus phase. The further exploration of the estrous cycle's impact on the ability of mice to be infected by *Neisseria gonorrhoeae*, particularly in the context of functional adhesion targets (hCEACAMs), has yet to undertaken.

Rationale, Specific Aims and Hypothesis

Neisseria gonorrhoeae is a gram-negative human pathogen responsible for the sexually transmitted infection (STI) gonorrhea. In men, gonorrhea manifests as simple urethritis, however in women, the infection can ascend into the upper genital tract and cause Pelvic Inflammatory Disease (PID) (Ghanem, 2015). PID is a highly inflammatory clinical condition that can cause chronic pelvic pain, infertility, and increased incidence of ectopic pregnancy (Ross, 2015). Although most cases are currently treatable with ceftriaxone/azithromycin dual therapy, GC has repeatedly demonstrated an ability to

rapidly develop antimicrobial resistance (Willcox, 1970; Mayfield, 1989; 2007; Workowski and Bolan, 2015). In fact, there have been multiple cases of fully drug resistant gonorrhea worldwide in recent years (Kirkcaldy et al., 2011; Ohnishi et al., 2011). As more treatment failures occur and fully resistant GC circulates, the relatively rare serious complications of infection will start to become much more common. To prevent this, there is a clear need for new anti-gonococcal pharmaceuticals. A vaccine that prevents colonization and transmission would be the most effective option as GC would be expected to eventually develop resistance to any new antimicrobial. Despite the threat of widespread completely drug resistant gonorrhea, logistical and biological hurdles have prevented the creation of such a GC vaccine (Zhu et al., 2011).

In the pursuit of an anti-gonococcal vaccine, different researchers have demonstrated the immunogenic potential of various gonococcal antigens; however no vaccine has shown protective efficacy and some have even resulted in greater risk of disease (Wetzler et al., 1992b; Ngampasutadol et al., 2006; Price et al., 2007; Jerse and Deal, 2013). From these unsuccessful attempts, we have learned that GC's high rate of antigen and phase variation allows for the bacterium to modulate the expression of an antigenic target, and that even if a vaccine induced antibody has a target to bind to, an anti-RMP response can undermine its bactericidal activity. These characteristics suggest a poly-antigenic vaccine may be needed so that the broad multi-target immune pressure applied would be too great for the gonococcus to avoid via expression changes, as well as the need for any vaccine to be pure of any RMP contamination.

A few studies have hinted at a protective anti-gonococcal response induced by vaccines using Outer-Membrane-Vesicles (OMVs) (Plante et al., 2000; Liu et al., 2018). These naturally occurring vesicles contain many outer membrane antigens in their native conformation in the context of potent immunostimulatory pathogen associated molecular patterns (PAMPs). These studies unfortunately utilized vesicles with high levels of contamination with cytoplasmic components and were not derived from an RMP lacking strain of GC. Additionally, the studies were carried out in the murine estrus model of infection. The estrus model requires β-estradiol and antibiotic treatment to create a permissive environment in the mouse vagina, allowing for a poorly inflammatory gonococcal colonization of the normally restrictive murine mucosal surfaces (Taylor-Robinson et al., 1990; Jerse, 1999). It is possible that this model may not reflect the biology of human infection enough to serve as a reliable proving ground for anti-gonococcal pharmaceuticals.

It has previously been shown that the gonococcus is so remarkably adapted to human biology that it is rendered non-viable in other organisms (Lee and Schryvers, 1988; Gray-Owen and Schryvers, 1993; Wang et al., 1998; Virji et al., 1999; Lee et al., 2007; Youssef et al., 2009; Voges et al., 2010). One of the primary restrictive interactions is the adhesion reaction between human CEACAMs and gonococcal Opa proteins (Voges et al., 2010). Human CEACAMs 1, 3, 5, and 6 mediate the adherence of gonococci to the mucosal surface of both the upper and lower female genital tract, in addition to mediating both pro-host and pro-bacterial immune mechanisms (Muenzner et al., 2000; McCaw et al., 2003; Muenzner et al., 2005; Sadarangani et al., 2011). Recently, transgenic mice that

express a collection of the neisserial-relevant hCEACAMs have been created and shown to recreate the tissue expression pattern seen in humans (Chan and Stanners, 2004; Gu et al., 2010).

Based on this body of literature **we hypothesize that the natural reproductive cycle as well as human CEACAM expression determine the natural course of infection in murine upper and lower genital tract *Neisseria gonorrhoeae* infection, and that an OMV vaccine derived from an *rmp* deletion mutant *Neisseria gonorrhoeae* strain can induce a broadly reactive anti-gonococcal immune response.** We tested this hypothesis through the following research aims:

Aim 1: Determine the impact of estrous cycle phase and human CEACAM expression on *Neisseria gonorrhoeae* infection kinetics, progression, and host response.

Aim 2: Isolate and characterize naturally released outer membrane vesicles from an *rmp* deletion mutant strain of *Neisseria gonorrhoeae*.

Aim 3: Examine the murine host response to an RMP-deficient nOMV *Neisseria gonorrhoeae* vaccine

We addressed these aims and underlying hypothesis through the following experiments. The studies of novel GC infection models were done in conjunction with Dr. Epshita Islam of Dr. Scott Grey-Owen's group at the University of Toronto. They

infected hCEACAM expressing mice and we characterized the host response to GC infection in different combinations of estrous cycle phase, hCEACAM expression and site of gonococci deposition. Since the current standard model of GC infection is the vaginally infected wild-type female mouse in estrus phase, we first examined the host response in those conditions to set a baseline. Through this approach we hoped to observe and describe the spectrum of disease that could be induced with our modern modeling tools and highlight novel conditions that recreate the symptomatic phases of human gonorrhea. As OMVs have not previously been described from an *rmp* deletion mutant strain of *Neisseria gonorrhoeae* we had to first establish an isolation protocol, characterize the resulting material and compare it to particles known to be OMVs from wild type bacteria. The results of the studies undertaken to evaluate these aims can be found in Chapters 3, 4, and 5 with a discussion of the general implications of the results found in Chapter 6.

Chapter 2: Materials and Methods

Chapter Three Methods

Bacterial strains

Neisseria gonorrhoeae used for experimental infection in studies involving only wild-type mice were low passage isolates originally collected during a longitudinal study of commercial sex worker in Nairobi, Kenya (Fudyk et al., 1999; Islam et al., 2016). For experiments utilizing hCEACAM expressing transgenic mice, all mice (even wild-type controls where applicable) were infected utilizing an MS11 strain of *Neisseria gonorrhoeae* that constitutively expresses Opa_{57}. This Opa form is known to bind to hCEACAM1, 3, 5, and 6 (McCaw et al., 2004). This strain is maintained by periodic subculturing based on phenotype and confirmation of proper Opa expressing by western blot (McCaw et al., 2004). All strains used are known to be resistant to the antibiotic treatment given to mice during experimental infections (see below). For all strains, bacteria were grown on GC agar (Becton Dickinson, Sparks, USA) supplemented with IsoVitalex (Becton Dickinson, Sparks, USA) at 37°C in a humidified 5% CO_2 atmosphere and prepared for infections as detailed below.

Animal strains

Wild-type 6-week old FvB mice were purchased from Charles River (Canada) and acclimated for 1-2 weeks before entering the experimental protocols.

The human transgenic hCEACAM1 (Gu et al., 2010) mice and CEABAC2 (Chan and Stanners, 2004) mice are both FvB background and were bred and housed in the

University of Toronto Division of comparative Medicine's Centre for Cellular and Biomolecular Research. Mice utilized in the presented experiments were 6-10 weeks of age.

All animal studies were reviewed and approved by the University of Toronto's Local Animal Care Committee (Permit #200010551) which operates under the ethical and legal requirements of the province of Ontario's Animals for Research Act and the federal Canadian Council on Animal Care (CCAC). Experiments were designed to reduce animal use and when unable to minimize or avoid suffering.

Reproductive cycle arrest by hormone treatment

Estrus arrest: Two days prior to infection, mice received a subcutaneous injection of water soluble β-estradiol (0.5mg/mouse, Sigma Aldrich, Oakville, Canada) to prolong estrus phase.

Diestrus arrest: To arrest/synchronize female mice in diestrus phase, they received DepoProvera (2mg medroxyprogesterone acetate/mouse, Pfizer Canada Inc., Quebec, Canada) 5 days prior to infection.

Reproductive cycle staging

Beginning 5 days before infection, mice reproductive cycle phase was evaluated daily by cytological analysis of wet mounts (Caligioni, 2009). Slides were prepared from 30 µl phosphate-buffered saline (PBS, Life Technologies, Burlington, Canada) vaginal washes and viewed under a 40x objective.

Vaginal infection

Mice were observed for ~5 days to determine estrous cycle stage. Once mice were in diestrus (Day -2), hormone and antibiotic treatments were started. Mice received subcutaneous injections of 0.5mg β-estradiol on days -2, 0, and 3. Intraperitoneal antibiotics (2.4mg Streptomycin sulfate + 0.6mg Vancomycin HCl in 200µl PBS) were injected once on day -2, twice on day -1 and once a day on day 0-5. Starting day -2, mice also received Trimethoprim (0.04g/100ml) in their drinking water. On day 0 (day of infection), an overnight lawn of *Neisseria gonorrhoeae* was collected into 1 ml of supplemented PBS (0.9mM $CaCl_2$, 0.5mM $MgCl_2$) (PBS^{++}, Life Technologies, Burlington, Canada). The concentration of bacteria in the resulting solution was calculated based on OD550 and additional PBS^{++} was used to achieve the proper concentration for infection. Just prior to infection, the vagina was washed 3x 30µl with PBS, and then 10^7 gonococci in 5µl was deposited in the vagina using a P10 pipette tip. Vaginal infections were performed on conscious mice. At the indicated time points after infection, mice were sacrificed by CO_2 asphyxiation. Sera were obtained via cardiac puncture. Lower and upper genital tract tissues were removed and separated at the point where the cervix joins the uterine body. Collected tissue was frozen using liquid nitrogen and stored at -80^oC until analyzed.

Murine transcervical Infection

A single strain culture of *Neisseria gonorrhoeae* was grown overnight on a chocolate agar plate to produce a lawn of bacterial colonies. A full plate was collected into 1 mL of PBS supplemented with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS^{++}, Life Technologies, Burlington, Canada). The OD_{550} absorbance of the bacterial suspension was measured and used to calculate the concentration of gonococci. This initial suspension was then diluted with PBS^{++} to produce a $5x10^8$ gonococci per milliliter suspension. Mice of known reproductive cycle phase were then anesthetized via inhalation of isoflurane. Infection was achieved as previously described (Islam et al., 2016). Briefly, anesthetized mice were laid prone at a 45-degree angle and, using a blunted 25-gauge needle, 20 μl of the infection suspension (10^7 gonococci) was delivered directly into the uterine horns. Six hours after infection, the mice were sacrificed by CO_2 asphyxiation. Sera were obtained via cardiac puncture. Lower and upper genital tract tissues were removed and separated at the point where the cervix joins the uterine body. Collected tissue was frozen using liquid nitrogen and stored at -80^{o}C until analyzed.

Tissue processing

Frozen tissue samples were thawed and divided evenly for protein or RNA extraction. Tissue processed for RNA was placed in TRIzol and homogenized using QIAshredder tissue homogenizer kits (Qiagen Cat#79654). RNA was extracted from the tissue homogenate using an RNeasy Mini kit (Qiagen Cat# 74104). Isolated nucleic acid

was initially analyzed for purity and integrity by 280/260 absorbance ratio via Nanodrop. Samples were then frozen at -80^{o}C until ready for use.

Tissue processed for protein was processed as follows. Individual tissue samples were pulverized using a scalpel blade, resuspended in 500 µl of tissue homogenization buffer (1X PBS containing 0.1% Triton, 5 mM EDTA, 1 mM PMSF, 2 µg/ml Aprotinin, and 1 µg/ml Pepstatin) then homogenized with a 5-mm stainless steel bead (Qiagen, Valencia, CA) at 500 oscillations/min for 15 min at 4°C using TissueLyser LT (Qiagen). Tissue debris was removed from solution by centrifugation for 10 min at 16,060 x g and 4°C. Supernatant was collected and the total protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA) using BSA as standard.

Cytokine ELISAs

Tissue levels of Il-1α, KC, and MIP-1α were evaluated using commercial cytokine ELISA kits from R&D systems (Cat#DY400-05, DY453-05, and DY450-05 respectively). For each ELISA, 100µg of extracted protein (as determined by BCA, see tissue processing methods) was evaluated for the concentration of a specific cytokine. Each sample was analyzed in duplicate. ELISAs were run following manufacturer's published methods. Data was analyzed and graphed in Prism.

LUMINEX Assay

Fifty micrograms (50µg) of total protein in 50 µl, extracted from tissue as previously described, was analyzed to determine levels of cytokines and chemokines

using a 20-plex mouse cytokine Luminex panel (Novex® Life Technologies, Carlsbad, CA), as per manufacturer instructions. Plates were read on Bio-Plex MAGPIX multiplex reader (Bio-Rad, CA) using xPONENT software (Life Technologies).

Microarray

RNA expression was profiled by the Boston University Microarray and Sequencing Resource using Affymetrix Mouse Gene 2.0 ST microarrays. Samples were processed in two batches of nearly identical size and representation of experimental groups to reduce any batch effect. Biotin labeling was performed using the WT Plus reagent kit (Affymetrix, Santa Clara, CA) according to the manufacturer's protocol. The labeled, fragmented DNA was hybridized to the Affymetrix Mouse Gene 2.0 ST Array for 18 hours in a GeneChip Hybridization oven 640 at 45°C with rotation (60 rpm). The hybridized samples were washed and stained using an Affymetrix fluidics station 450. After staining, microarrays were immediately scanned using an Affymetrix GeneArray Scanner 3000 7G Plus.

Quality assessment

Prior to analysis of expressional data, the quality of the microarrays was assessed using two metrics: Relative Log Expression (RLE), which indicates whether the distribution of intensity values of a relatively dim array have been artificially skewed upwards by the Robust Multiarray Average (RMA) normalization algorithm, and Normalized Unscaled Standard Error (NUSE), which is a measure of the noise inherent in

the estimate of each probeset (gene). The median RLE values were relatively similar across 13 of the samples (range -0.05 to 0.05), as were the median NUSE values (range 0.99 to 1.02). However, the remaining two samples (GC-infected estrus phase samples 6 and 7) had higher median RLE (0.092 and 0.105, respectively) and NUSE (1.03 and 1.04, respectively) values, indicating that these two arrays may be of lower quality compared to the rest of the experiment.

Assessment of and correction for array batch effect

Because the arrays were processed in two separate batches, Principal Component Analysis (PCA) was employed to assess the strength of batch effect (Figure 2.1a). The samples cluster primarily by reproductive-cycle phase, but separate within each phase primarily by batch, indicating that a substantial batch effect is initially present. In order to correct for this effect, expression values were adjusted using the ComBat algorithm, and PCA was repeated (Figure 2.1b). Following batch adjustment, the samples again separate well by reproductive-cycle phase, but within the diestrus phase group, greater separation by treatment was seen. The GC-infected estrus samples 6 and 7, which had been identified as being of lower quality, still separated from GC-infected estrus samples 4 and 5 along the PC2 axis, indicating that batch adjustment did not fully account for the relative difference in quality between these two pairs of samples. Despite persistence of moderate batch effect, samples 6 and 7 were retained for analysis, since their median RLE and NUSE values were not drastically higher than the rest of the arrays, and without them, batch one GC-infected estrus arrays would be unopposed by any batch two arrays.

The potential loss of array sensitivity due to remaining batch effect, is outweighed by the larger effect of an unopposed batch effect. In addition, the phenotypic differences described in this model suggests that there are major differences in induction of biological pathways that may still be identified even in a slightly less sensitive system. Indeed, significant transcriptional differences were identified indicating a non-critical impact by the residual batch effect following ComBat adjustment.

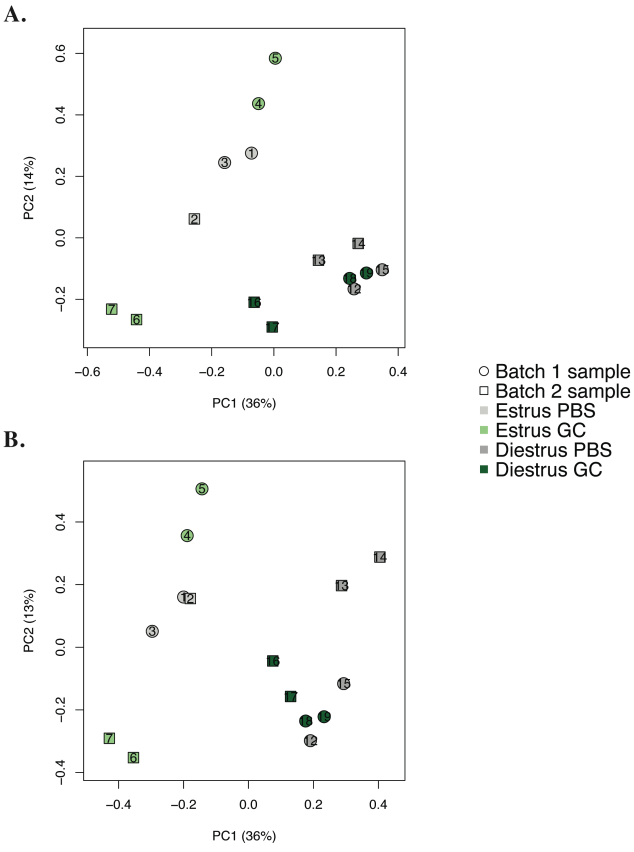


Figure 2.1: Principal Component Analysis (PCA) before and after batch correction. All samples are plotted with respect to the first and second Principal Components (PC), computed using log2 (expression) values *z*-normalized across all samples (to a mean of zero and a standard deviation of one). PCA was performed both prior to (**A**) and following (**B**) correction for array batch effect using ComBat. Light and dark colors indicate estrus-phase and diestrus-phase samples, respectively, and gray and green indicate PBS-treated and GC-infected samples, respectively. Samples from array batches 1 and 2 are plotted as circles and squares, respectively. N=4 for all groups except PBS Estrus N=3

Microarray analysis

Mouse Gene 2.0 ST CEL files were normalized to produce gene-level expression values using the implementation of the Robust Multiarray Average (RMA) (Irizarry et al.,

2003) in the affy package (version 1.36.1) (Gautier et al., 2004) included in the Bioconductor software suite (version 2.12) (Gentleman et al., 2004), and an Entrez Gene-specific probeset mapping (17.0.0) from the Molecular and Behavioral Neuroscience Institute (Brainarray) at the University of Michigan (Dai et al., 2005). Array quality was assessed by computing Relative Log Expression (RLE) and Normalized Unscaled Standard Error (NUSE) using the affyPLM package (version 1.34.0). The implementation of the ComBat algorithm in the sva package (version 3.4.0) was used to adjust the expression values for the batch in which the arrays were scanned, adjusting for phase, infection, and the interaction of the two (phase:infection) as covariates. Differential expression was assessed using the moderated (empirical Bayesian) t test implemented in the limma package (version 3.14.4) (i.e., creating simple linear models with lmFit, followed by empirical Bayesian adjustment with eBayes). Correction for multiple hypothesis testing was accomplished using the Benjamini-Hochberg false discovery rate (FDR). Human homologs of mouse genes were identified using HomoloGene (version 68) (Coordinators, 2013). All microarray analyses were performed using the R environment for statistical computing (version 2.15.1).

Gene Set Enrichment Analysis

GSEA (version 2.2.1) (Subramanian et al., 2005) was used to identify biological terms, pathways and processes that are coordinately up- or down-regulated within each pairwise comparison. The Entrez Gene identifiers of the human homologs of the genes interrogated by the array were ranked by the *t* statistic computed between Ng and PBS

within each reproductive-cycle phase, or by the treatment or phase:treatment *t* statistic. Mouse genes with multiple human homologs (or *vice versa*) were removed prior to ranking, so that the ranked list represents only those human genes that match exactly one mouse gene. This ranked list was then used to perform pre-ranked GSEA analyses (default parameters with random seed 1234) using the Entrez Gene versions of the Hallmark, Biocarta, KEGG, Reactome, Gene Ontology (GO), and transcription factor and microRNA motif gene sets obtained from the Molecular Signatures Database (MSigDB), version 5.0 (Subramanian et al., 2007).

Ingenuity Pathway Analysis (IPA)

Canonical pathways were automatically identified using the 416 genes with FDR $q < 0.25$ for the phase:infection interaction *t* test (computed after removing genes that were not expressed above the median value of at least one array). Analysis was performed using IPA's reference database and all Ingenuity-supported third-party databases, set to consider direct molecular relationships, allowing for experimentally observed and predicted relationships with high confidence, and restricted to mouse tissue and cell lines. Differential fold changes were calculated by computing fold changes (infected versus uninfected) within each reproductive cycle phase and then obtaining the ratio of the two (diestrus:estrus). The granulocyte adhesion and diapedesis pathway figure was built using IPA Path Designer with differential infection-induced fold change data overlaid.

Chapter Four Methods

Neisseria gonorrhoeae growth

Briefly, MS11 strain *N. gonorrhoeae* (either wild-type or a Δ*rmp* mutant) was grown on chocolate agar plates (Remel, Cat#R01300) overnight at 37°C and 5% CO_2. Bacteria was transferred from the plate to liquid GC broth (1.5% Protease peptone 3 w/v, 22.96mM K2HPO4, 7.35mM KH2PO4, 85.62mM NaCl, 1% Isovitalex v/v) by a sterile nylon swab to obtain an initial OD_{600} of approximately 0.1 as measured by spectrophotometry (Biorad SmartSpec 3000, Cat#170-2501). Initially four, 20 mL liquid cultures, were grown in vented 50 mL conical tubes (Corning mini bioreactor, Corning, Cat#CLS431720; Corning, NY) in a shaking incubator set at 37°C and 180 rpm. After cultures reached an OD_{600} of approximately 0.6 (around 4 hours of growth) each 20-mL culture was used to seed a 125-mL culture to an OD_{600} of approximately 0.1. These 125 mL cultures were allowed to grow in a shaking incubator at 37°C and 180 rpm until they reached an OD_{600} of approximately 0.6 (~4 hours growth). The cultures were then used to evenly seed 3000 mL of 1% isovitalex supplemented GC media separated into two sterile 2800 mL bacterial culture flasks. This final growth volume was allowed to grow overnight in a shaking incubator set to 37°C and 160 rpm.

Neisseria gonorrhoeae natural outer membrane vesicle (nOMV) isolation

nOMVs were isolated from *Neisseria gonorrhoeae* using a modified version of an established *Neisseria meningitidis* nOMV isolation protocol (Pajon et al., 2013). All nOMVs, despite specific source strain used, were isolated using the same protocol.

Briefly, nOMVs were isolated from 3-liters of bacterial culture that had been maintained in log-phase growth over the course of 24 hours. To begin, the entire culture volume was collected and split evenly among six 500 mL sealable centrifuge tubes (Beckman Coulter, Cat#355607). Bacteria and large debris were removed from solution through centrifugation; 4,000 g for 30 minutes at 4^{o} C (Rotor; Beckman Coulter JA-10 Cat#369687) (Centrifuge; Beckman Coulter, Avanti J-E Cat#A20698). Following centrifugation, the low speed supernatant was carefully removed and filtered with a 0.45 µl PES filter (CellTreat, Cat#229713) to sterilize and further remove large debris. Approximately 600 mL of sterile 5x nOMV buffer (15% Sucrose, 1M Glycine pH 8.0) was then added to the low speed supernatant to provide osmotic support for the vesicles. Sterility of the low speed supernatant was confirmed by overnight culture of 200 µL on a chocolate agar plate. The low speed supernatant was kept in a sealed sterile bottle(s) at 4^{O}C while sterility was confirmed.

After confirming sterility of the low speed supernatant, vesicles were concentrated by stirred ultrafiltration. Solution was filtered through a 100,0000 NMWL membrane mounted in a 400 mL Amicon ultrafiltration cell (Millipore, Cat#UFSC40001) by constant 10 psi pressure from medical grade Nitrogen. Once the volume of low speed supernatant reached a volume of approximately 50 mL the filtration cell was depressurized, additional supernatant was added, and the cell was repressurized and filtration continued. This was repeated until all ~3600 mL of solution was concentrated to approximately 100 mL. of retentate. The concentrated nOMVs were then removed from the retentate by ultracentrifugation in reinforced high-speed ultracentrifuge tubes;

100,000 g for 2 hours at 4^{O}C (Tubes; Beckman Coulter, Cat# 355631) (Rotor; Beckman Coulter, SW-28 Cat#342204) (Ultracentrifuge; Beckman Coulter, Optima XPN 100k Cat#A99846). Following centrifugation, the supernatant was removed from the nOMV pellet gently and discarded. A small volume of 1x nOMV buffer (3% sucrose, 0.2 M glycine, pH 8.0) was then placed on top of the nOMV pellet and the vesicles were allowed to enter solution while stored overnight at 4^{O}C. The nOMV colloid was then consolidated into one sterile screw-top cryogenic vial and <0.001% Sodium azide was added to prevent fungal growth. The nOMV preparation was then stored at 4^{O}C and maintained sterile during all subsequent uses.

Evaluation of isolated nOMV size by Dynamic Light Scattering (DLS)

Effective particle diameter was estimated by Dynamic Light Scattering, using a Brookhaven 90 plus Nanopartical Analyzer (Brookhaven Instruments, Holtsville, NY). Briefly, the analyzer was turned on and the laser was allowed to warm up for ~15minutes. Brookhaven Instrument Corporation's Particle Sizing Software was used to collect data. Approximately 1 mL of nOMV suspension was transferred to a cleaned, 1.5 mL polystyrene cuvette (Fisher Scientific, Cat#14-955-127). Default parameters were used: Temperature: 25^{o}C, Suspension: Water, Viscosity: 0.89cp, Ref. Index Fluid: 1.330, Angle: 90.00, Wavelength: 657nm, Dust cutoff: 90, Run duration: 30 seconds. A total of 5 runs were completed and results were averaged. A table of observed effective diameters as well as a histogram were exported from the software. The sample was collected and the machine shut down.

Protein quantification

Protein concentration of nOMV associated protein or any other protein source was determined by cold Acetate extraction/purification of protein followed by Pierce Modified Lowry protein assay (ThermoFisher Scientific, Cat#23240) per manufacturer's instructions.

Immunostimulation assay by Human embryonic kidney (HEK) cells

The ability of nOMVs to interact and stimulate cells through the Toll-Like-Receptor (TLR) homodimer TLR4/TLR4 and heterodimer TLR1/TLR2 was evaluated using HEK cells designed to overexpress those receptors in isolation and a control cell line that was transfected with a non-coding vector (pcDNA) (Chow et al., 1999). Briefly, cells of a given genotype were plated in a 24-well plate at a density of 0.5×10^5 cells per well and allowed to reach confluency (typically 24hrs). Once confluent, each well was treated for 24-hours with a single compound at a given concentration; 10μg/ml nOMV, 1μg/ml nOMV, 0.1μg/ml nOMV, LOS 100ng/ml, P1B 10μg/ml, TNF-α 20ng/ml, and an untreated control. Following the 24hr stimulation, supernatants were collected and evaluated for Il-8 concentrations by ELISA (BD OptEIA, Becton Dickson) per manufacturer's protocol.

Electron Microscopy Grid preparation and sample staining

Formvar support films (in house preparation per Bullitt lab protocol) were adhered to copper transmission electron microscopy grids (EM Science, Cat.#0300-Cu) and then coated with atomized carbon from carbon rods using a vacuum evaporator (Denton Vacuum Evaporator, Model #DV502A) and stored until needed. Immediately prior to grid use, they were cleaned by two 30-second plasma discharges under a near vacuum (~0.08mBar) in a glow discharge apparatus (Balzers/Baltech Glow Discharge Apparatus, Model# CTA010) set at 180V. The grids were then immediately used for sample adhesion and staining. Briefly, a small volume of the sample of interest (OMVs from a single isolation) was diluted, if necessary, to obtain a protein concentration of no more than 2 μg protein per 1 μl of suspension. To adhere the sample to the grid, 4 μl of sample was placed upon the recently cleaned Carbon coated face of the grid and allowed to incubate for 1 minute. The drop of sample was then removed from the grid using filter paper gently placed perpendicular to the grid's face along its side for approximately 5 seconds or until the liquid is completely removed from the grid. The grid was then washed with a drop of 0.2 μm filtered ultra-pure deionized water that was then immediately removed via gentle suction from a pasture pipette. Without allowing the grid to completely dry, it was washed a total of 12 times with 12 drops of water. Any remaining water was then removed from the grid, using the same perpendicular blotting technique used above, with a clean piece of filter paper. The grid was then stained with one drop of a ~2% Uranyl-Acetate negative stain (Prepared in house from radioactively depleted powder (Tod Pella, Cat.#19481) in water and brought to a final pH of 4.4) for

one minute. Stain solution was removed from the grid with a clean piece of filter paper via the same perpendicular blotting technique described before, this time blotting for 10 seconds. The grid was then allowed to fully air dry for approximately 3 minutes before being placed in a protective grid case for subsequent examination.

nOMV Visualization by Transmission Electron Microscopy

Negative stained samples were visualized by transmission electron microcopy using a Phillips CM12 Transmission Electron Microscope configured with a lanthanum hexaboride (LaB_6) crystal electron source set to an accelerating voltage of 120 KV and liquid Nitrogen cold finger cooled chamber. Images were captured using either; TVIPS TEITZ (Gauting, Germany) 1Kx1K CCD camera using EMmenu software (Version 4.0.9.83), TVIPS TEITZ TemCam F216 2Kx2K CCD camera using the same software, or by the built-in plate-camera (Phillips CM12) on electron image film (Kodak, SO-163 Film) developed according to manufacturer protocol and solutions. Physical films were digitized by high definition scanning performed by Colortek (Boston, Massachusetts).

Measurement of vesicle membrane thickness

Vesicle membrane thickness was measured by ImageJ. Using a 43,750x UA-negative stained TEM electron micrograph, thickness was measured in 4 places on 10 vesicles with outer and inner membrane surfaces clear and in focus. The 4 measurements for each vesicle were then averaged to obtain that vesicles average membrane thickness.

Data from the 10 vesicles from both Δ*rmp* and wild-type vesicles were analyzed by unpaired *t*-test and graphed in Prism.

Measurement of vesicle diameter

Vesicle diameter was measured in ImageJ. For each vesicle isolation, two 43,750x UA-negative stained TEM electron micrographs, taken from separate grid-squares, were examined. Every vesicle in the micrographs that had clear, in-focus outer membrane frontiers, and did not demonstrate alterations in shape do to external structures (i.e. other vesicles or grid bars) was measured. For each vesicle, two perpendicular measurements from outer membrane limit to outer membrane limit in taken (forming an "X" across the vesicle). The two diameter measurements were averaged to estimate vesicle diameter. For the four isolations, between 53 and 67 vesicles fit the criteria outlined above and were measured. The data was analyzed by one-way ANOVA and graphed in Prism.

Polyacrylamide gel electrophoresis separation of structures

Protein/Lipooligosaccharide (LOS) was prepared for gel electrophoresis by initial concentration. Briefly, associated structures were extracted from samples by diluting it 1:4 in cold acetate and incubating at -20^{o}C for an hour. Following incubation samples were spun at 10000rmp for 10m at 4^{o}C to pellet precipitated structures. The pellet was allowed to dry and then resuspended in 10µl loading buffer (5% β-mercaptoethanol, 100mM Tris-Cl pH 6.8, 4% w/v sodium dodecyl sulfate (SDS), 0.2% w/v bromophenol blue). Samples were boiled for 5 minutes immediately before loading into wells of a

polyacrylamide gel. Gels were 15% polyacrylamide, 10% SDS. Samples were run at 60-volts for 20 minutes to concentrate structures in the stacking gel, and then for 90 minutes at 120-volts to separate them in the resolving gel. Gels were then processed by coomassie blue staining to visualize proteins, or silver stain to visualize LOS.

Staining of PAGE gels with Coomassie blue

Coomassie blue solution was prepared according to the manufacturer's instructions by mixing equal volumes dH_2O and methanol, adding 10% acetic acid and dissolving 0.25% w/v Coomassie Brilliant Blue (Sigma). To facilitate dissolving the coomassie, the solution was sonicated and then remaining particles removed by filtration with Whatman 3MM filter paper (Whatmam, UK).

Following gel electrophoresis, a gel was rinsed with dH_2O, drained, then placed in enough Coomassie liquid stain so that it was completely covered. The gel was allowed to stain at room temperature for 4 hours on a rocker. After 4 hours, the coomassie was drained from the gel which was then washed 4 times with dH_2O. The gel was then incubated in destaining solution (25% propanol, 10% acetic acid in dH_2O) until background staining was removed and protein bands were clear (typically took an ~12hr overnight incubation). Gels were imaged using a Biorad Gel Doc XR imaging system (Biorad, USA) and then dried on a frame (Owl Separation System, NH) between two sheets of drying paper (Promega, WI, USA) per manufacturer's protocol. For figure 4.6, the coomassie stained protein banding pattern was interpreted by ImageJ by selecting each lane individually using the "Select First Lane" and then "Select Next Lane"

functions. Lanes were selected from the bottom of the stacking gel to the edge of the loading dye frontier. Once all lanes were selected, the histogram was generated by the "Plot Lanes" function. The resulting histogram was annotated with ladder band masses in Adobe Illustrator.

Silver staining of PAGE gels for LOS

Gels with known amounts of nOMV protein (as well as a positive control with known quantity of LPS) were run through gel-electrophoresis as previously described. Following PAGE, the gel was placed in a glass petri dish along with 50 mL of fixative solution #1 (40% methanol, 10% acetic acid in dH_2O) for 30 minutes. Fixative #1 solution was then drained, replaced with 50 mL of fixative solution #2 (10% ethanol, 5% acetic acid in dH_2O) and allowed to sit at room temperature for 15 minutes. The first volume of fixative #2 was then drained and replaced with another 50 mL and the gel was fixed for an additional 15 minutes. After the second incubation with fixative #2 was finished, the solution was completely drained and 50 mL of oxidizer solution (prepared following the Bio-Rad Silver Stain kit's instructions) was place in the petri dish and allowed to sit for 5 minutes. After the 5-minute oxidizing step, the gel was rinsed with repeated 5 minute incubations in dH_2O until the gel loses all of the yellow/orange coloration from the oxidizer solution. Once the final water wash was removed, 50 mL of Silver Stain reagent (prepared following Biorad kit instructions) was placed on the gel and allowed to react for 20 minutes. After the silver stain reagent step, the solution was poured off and the gel was quickly rinsed for a minute with dH_2O. After the water rinse

was removed, ~25 mL of developer (prepared following Biorad kit instructions) was placed on the gel and then immediately removed after ~30 seconds. Fresh developer was then added to the dish only to be removed once it had become cloudy at which point it was removed and replaced with fresh developer solution. This process of developer solution exchange was continued until a strong visible band appeared in the positive control lane. Once bands were clearly visible, the last volume of developer was removed and replaced with a 5% acetic acid in water, stop-solution and the gel was allowed to sit for 5 minutes. The gel was then rehydrated by ~1-hour incubation in dH_2O and then imaged and dried as previously described in the Coomassie blue methods section.

Animals

Wild-type 6-8-week-old female C57/BL6 mice utilized in nOMV vaccination experiments were purchased from Jackson Laboratories (Stock #000664). These mice were housed in Boston University School of Medicine's Laboratory Animal Science Center (LASC); an Association for Assessment and Accreditation of Laboratory animal care internationally accredited facility. All experiments using animals were reviewed and approved by Boston University School of Medicine's (BUSM) Institutional Animal Care and Use Committee (IACUC) (Protocol#AN-15593.2016.09). All efforts were made to reduce animal numbers and where possible to reduce and eliminate any pain or suffering. All animals were allowed to acclimate to the animal facility for 2 weeks after arrival before being used in experiments.

nOMV immunizations

Following a 2-week acclimation period, 8-10-week-old female C57/BL6 mice were separated into cages based on treatment group (no more than 5 mice per cage, multiple cages per treatment group was used if necessary). On the day prior to the first immunization, blood and vaginal secretions were collected as follows. Mice were placed in a tailveiner restrainer and a small cut was made on the dorsal side of their tail as distal from the base as possible with a sterile blade. Approximately 200ul of blood was collected in a microcentrifuge tube containing 10µl 1000-Units/ml heparin sulfate. Pressure was maintained on the incision site with sterile gauze for ~15 seconds to encourage clotting and then the mice were returned to their cage. Whole blood was processed for sera by subsequent centrifugation for 10minutes at 10,000rmp at 4^{o}C. Sera was then pipetted off the pellet, aliquoted and froze for future use.

For vaginal secretions, mice were anesthetized with isoflurane. Induction was performed in an anesthesia induction box with 5% isoflurane O_2 mixture. The mice were then maintained in anesthesia with a 2% Isoflurane mixture delivered through a nosecone at ~0.8L/minute. While under anesthesia, the body temperature of the mice was maintained using a covered chemical heating pad. To collect vaginal secretions, anesthetized mice were placed in a supine position and immobilized with gentle pressure on the tail. The vagina was then washed gently 3 times with 30µl sterile PBS containing 1x protease inhibitors (Roche, cOmplete EDTA-free protease inhibitor cocktail, Cat#11836170001) delivered by a sterile P20 pipette tip. That initial 30µl wash was collected and using a new clean tip, another 30µl was used to wash the vagina in the

same manner. This was repeated for a grand total of 5 washes collecting ~150µl of solution. The mice were then allowed to recover from anesthesia alone in a clean cage before being returned to their home cage.

The next day, mice were immunized according to their group. For nOMV vaccines, isolated sterile Δ*rmp* nOMVs were diluted in sterile PBS to the desired concentrations. Control mice were immunized with normal sterile PBS. Subcutaneous (SC) immunizations were performed as follows. Mice were immobilized using the tailveiner and injected with the desired dose of nOMVs in 100µl just below the skin along the back above the tail. Mice were then returned to their home cage. For intranasal (IN) immunizations, mice were anesthetized with Isoflurane exactly as described for vaginal washes. The anesthetized mouse was then gently immobilized in the hand by grabbing the skin at the neck and the lower body. As the mouse was held at a ~30^{O} angle (head up) a P20 was used to deliver 10µl of nOMV vaccine into each nostril (20µl total per dose). The mouse was held at this angle until the solution had fully entered the nasal passages, it was then placed in a clean cage by itself to recover and then moved to its home cage.

On days 13 and 27 after the first immunization, blood from all mice was collected exactly as previously described. On days 14 and 28 of the experiment, mice received either IN or SC vaccines, as appropriate for their treatment group, exactly as previously described. Finally, on day 42, serum and vaginal secretions were collected from all mice as previously described and mice were sacrificed by CO_2 asphyxiation and cervical dislocation.

Antigen specific Immunoglobulin ELISA

For both nOMV and *Neisseria gonorrhoeae* specific ELISAs, the same general protocol was followed as has been previously published with the following alterations (Liu et al., 2008; Platt et al., 2013). First, 4HBX hydrophilic treated flat-bottom 96 well plates (ThermoFisher Sci., Cat#3855) were coated. For both antigen specific ELISAs, an OD-value:IgG-concentration standard curve was constructed based on a full plate of standards. The standard plate was coated with 100µl of 10 µg/mL $F(ab')_2$ Fragment Goat anti-mouse IgG (Jackson ImmunoResearch, Cat#115-006-006) in carbonate buffer. For nOMV ELISAs, plates were coated with 100µl of nOMV associated protein at a concentration of 5 µg/mL in carbonate buffer. For GC specific ELISAs, wild-type MS11 strain *Neisseria gonorrhoeae* was grown for 8 hours in GC-media (see bacterial growth methods for details). Cells were then pelleted, resuspended in carbonate buffer and 100µl were placed in each well of 96 well plates at a density of $5x10^6$ gonococci/well. Plates were allowed to coat overnight at 4^oC.

Following overnight coating, the plates were washed 12 times by the placement and aspiration of ~250µl ELISA wash buffer (0.05% Tween in PBS). After aspiration of the final volume of wash buffer, each well was loaded with 200µl of blocking buffer (5% Bovine Serum Albumin, 0.05% Tween in PBS), plates were covered and placed on a plate shaker for 2 hours. After the blocking step, plates were washed as previously described, and then loaded with 200µl of known dilutions of experimental samples into

antigen coated wells and known concentrations of mouse IgG (Sigma, Cat#15318) into $F(ab)^2$ coated wells. Plates were then covered and allowed to incubate overnight at 4^oC.

After overnight capture incubation, samples/standards were removed and wells were washed 12 times with wash buffer as previously described. Plates were then filled with 100µl per well of a 1:30,000 dilution of Alkaline phosphatase-conjugated Goat-anti-mouse IgG antibody (Sigma, Cat#A3438), covered, and placed on a shaker for 3 hours at room temperature. Following the 3-hour incubation with secondary antibody, the wells were washed 12 times as previously described, plates were filled with 100µl/well 1-step *p*-nitrophenyl phosphate (PNPP) (ThermoScientific, Cat#37621), covered and placed on a shaker for 30 minutes. After 30 minutes, the reaction was stopped by adding 50 µl 2N sodium hydroxide to each well. Absorbance of 405nm light was then immediately read using an ELISA plate reader (BioTek Synergy HT, BioTek) and data was captured using BioTek Gen3 software.

Western blotting (Immunoblot)

Proteins associated with Δ*rmp* nOMVs were precipitated via cold acetone extraction as described in the PAGE methods section. Exactly 10µg nOMV associated protein was loaded and separated by PAGE as previously described on a 15% polyacrylamide gel. The gel was then placed in transfer buffer (1x TGS, 20% v/v ethanol in dH_2O) to prevent drying while PVDF membrane was prepared. A section of PVDF membrane (GE Healthcare, Amersham Hybond 0.2µm membrane Cat#10600057) slightly larger than the polyacrylamide gel was cut and equilibrated in pure methanol for

5-minutes. While the membrane equilibrated, sections of whatman paper and western-blot sponges were immersed in transfer buffer. After the membrane was equilibrated the full transfer cassette was assembled as follows: Positive cassette plate, sponge, whatman paper, PVDF membrane, polyacrylamide gel, whatman paper, sponge, negative cassette plate. The cassette was assembled while immersed in transfer buffer to prevent the formation of bubbles between layers. The cassette was placed in a transfer box full of transfer buffer and surrounded by ice. Proteins were transferred from the gel to the membrane at 200mA (60V) for 1-hour.

After protein transfer, the membrane was equilibrated in TBS-T (0.01% Tween) for 5 minutes and then blocked (5% non-fat dry milk (NFDM) in TBS-T) for 1 hour. After blocking the membranes were probed with immune sera (pooled sera diluted 1:1000 in 5% NFDM in TBS-T) overnight at 4^{o}C on a rocker to prevent drying. The next day, primary antibody solution was removed and the membrane was rinsed three time for 5 minutes with 5% NFDM in TBS-T. Following the last wash, the membrane was put in horseradish-peroxidase conjugated horse anti-mouse IgG (Cell signaling, Cat#7076S) secondary antibody solution (1:5000 dilution in 5% NFDM in TBS-T). Membranes were incubated in the secondary antibody solution for 1 hour on a rocker at room temperature. After an hour, the membranes were removed and washed 6 times for 5 minutes each. The first 3 washes were with TBS-T and the final three were in TBS. After washing, ECL (GE Healthcare, Cat#RPN2232) solution was placed on top of the protein side of the membrane and allowed to react for 5 minutes. Following the 5-minute period, the ECL was removed by blotting with whatman paper. The immunoblots were then exposed to

Amersham Hyperfilm™ (GE Healthcare, Cat#28906837) for periods of time ranging from 10 seconds to 2 minutes. Films were then developed on a Kodak X-OMAT 2000A X-ray film processor. Images of the films were captured using a Biorad Gel Doc XR imaging system (Biorad, USA).

Chapter 3: Murine estrous cycle phase and human-CEACAM expression moderates the magnitude of host response to *Neisseria gonorrhoeae* infection

Introduction

In response to the looming threat of wide-spread fully antimicrobial resistant *Neisseria gonorrhoeae*, both the CDC and the WHO have identified a critical-level need for the development of novel anti-gonococcal pharmaceuticals (CDC, 2012; Carmeli, 2017). The successful development of these next-generation products will require *in vivo* testing that, at least initially, will need to occur in animal models.

Since its introduction in 1999, the female lower-genital tract vaginal colonization model has been the primary tool for *in vivo* experimentation with GC (Jerse, 1999; Jerse et al., 2011). Notably, bacteria are rapidly cleared during the diestrus phase of the murine reproductive cycle which means the model mouse must be artificially arrested in the estrus phase, and GC viability is additionally facilitated by suppression of the natural vaginal microbiome using antibiotics (Jerse, 1999). This estrus infection model displays a significant, albeit mild, induction of pro-inflammatory cytokines and influx of neutrophils following infection (Jerse, 1999; Song et al., 2008; Jerse et al., 2011). This well-established model is, therefore, reminiscent of the asymptomatic colonization in women rather than active lower genital tract infection or PID.

As in humans, it is possible that the reproductive cycle could influence the natural progression of infection in mice. In the original estrus model publication, a resistance to infection was described in diestrus (Jerse, 1999) however it is impossible to determine if that was the result of diestrus-related physiology or due to the lack of viable virulence

factor targets. The mild pathology (compared to human infection) of the estrus model and resistance to infection in diestrus may be the result of *Neisseria gonorrhoeae*'s significant human specific adaptations (Lee and Schryvers, 1988; Gray-Owen and Schryvers, 1993; Voges et al., 2010).

Work on the host-pathogen relationship of the gonococcus has identified the failure of its critical virulence factors to engage with and exploit the non-human forms of virulence factor targets as a major cause of its host specificity. As described previously, hCEACAMs are the target of the gonococcal adhesion mediating Opa proteins (Popp et al., 1999). In non-humans the bacterium cannot bind to CEACAMs and is unable to adhere to the mucosal epithelia and initiate the process of colonization and infection (Edwards and Butler, 2011). Additionally, hCEACAMs seem to mediate both active anti-bacterial (Hauck et al., 1998; Williams et al., 2000) and host-detrimental processes (Muenzner et al., 2005) in GC infection. These substantial differences in physiology are not addressed and overcome by the current estrus mouse model of GC infection. *This suggests that, even though the estrus model is permissive to colonization, its physiology may be different enough that the mechanisms underlying protective immunity in the model may not be the same mechanisms required for human protection.*

In an effort to better mimic human infection and responses to infection, several groups have started designing and creating transgenic mouse lines which express the human forms of molecules targeted by pathogen virulence factors. In the case of pathogenic *Neisseria* models, hCEACAM expressing mice have been produced. In the case of the hCEACAM1 mouse, the human gene, including the human promoter, was

introduced into the mouse genome. The resulting hCEACAM1 mouse demonstrated human-like expression of hCEACAM1 among the broad categories of tissues that were examined (Gu et al., 2010). A second, poly-transgenic, hCEACAM mouse was constructed by another group in an effort to recapitulate a greater portion of the hCEACAM dependent biology in infection (Chan and Stanners, 2004). This mouse, named CEABAC2 by its creators, expresses the hCEACAMs 3, 5, 6, and 7. Like the hCEACAM1 mouse, the CEABAC2 mouse demonstrated physiologic expression levels and each of the 4 genes were found to be generally expressed in tissues similar to humans (Chan and Stanners, 2004). Unfortunately, neither of these publications reported on the pattern of hCEACAM expression in the lower and upper FGT of the transgenic mice. Additionally, the natural human distribution of hCEACAMs in the human FGT wasn't well known.

Fortunately, our collaborators, the Gray-Owen lab, were able to elucidate not only the expression pattern in the transgenic mice, but compare it to hCEACAM expression in normal human FGT tissue (Islam et al., 2018). Figure 3.1 shows hCEACAM expression distribution in the hCEACAM1 mouse and the hCEACAM 3, 5, 6 expressing CEABAC2 mouse by immunohistochemistry using an antibody that reacts only to hCEACAMs. As hCEACAM1 mice are only transgenic for this single human CEACAM, we can interpret the positive staining of the uterine epithelia as indicative of hCEACAM1 expression on the apical side of uterine epithelial cells. In the same publication, the Gray-Owen group reported the presence of hCEACAM1 on various leukocytes as well. This strongly reflects the expression pattern that was found in humans in the same study (Table 3.1)

(Islam et al., 2018). The CEABAC2 mice expressed both hCEACAM5 and 6 in the squamous epithelia of the vagina and ectocervix which mirrors the ectocervical expression of hCEACAM5 in humans (Table 3.1). This along with the hCEACAM1's uterine restriction allows for the specific interrogation of epithelial hCEACAMs effect in upper or lower FGT infection. CEABAC2 mice also have hCEACAM3 expressing neutrophils which, at least *in vitro,* are known to have powerful anti-GC function due to this decoy receptor (Table 3.1)(McCaw et al., 2003; Sintsova et al., 2014).

Genotype	Vaginal epithelial cells	Uterine epithelial cells	Neutrophils	Macrophages and T-cells
FvB	-	-	-	-
hCEACAM1	-	hCEACAM1	hCEACAM1	hCEACAM1
CEABAC2	hCEACAM5 hCEACAM6	-	hCEACAM3 hCEACAM6	-
Humans	hCEACAM5	hCEACAM1	hCEACAM1 hCEACAM3 hCEACAM6	hCEACAM1

Table 3.1: Expression pattern of hCEACAMs in the FGT and leukocytes of transgenic mice reflect that of humans. This table is a summary of hCEACAM expression distribution within the female genital tract as reported in (Gu et al., 2010; Muenzner et al., 2010; Sintsova et al., 2014; Islam et al., 2018)

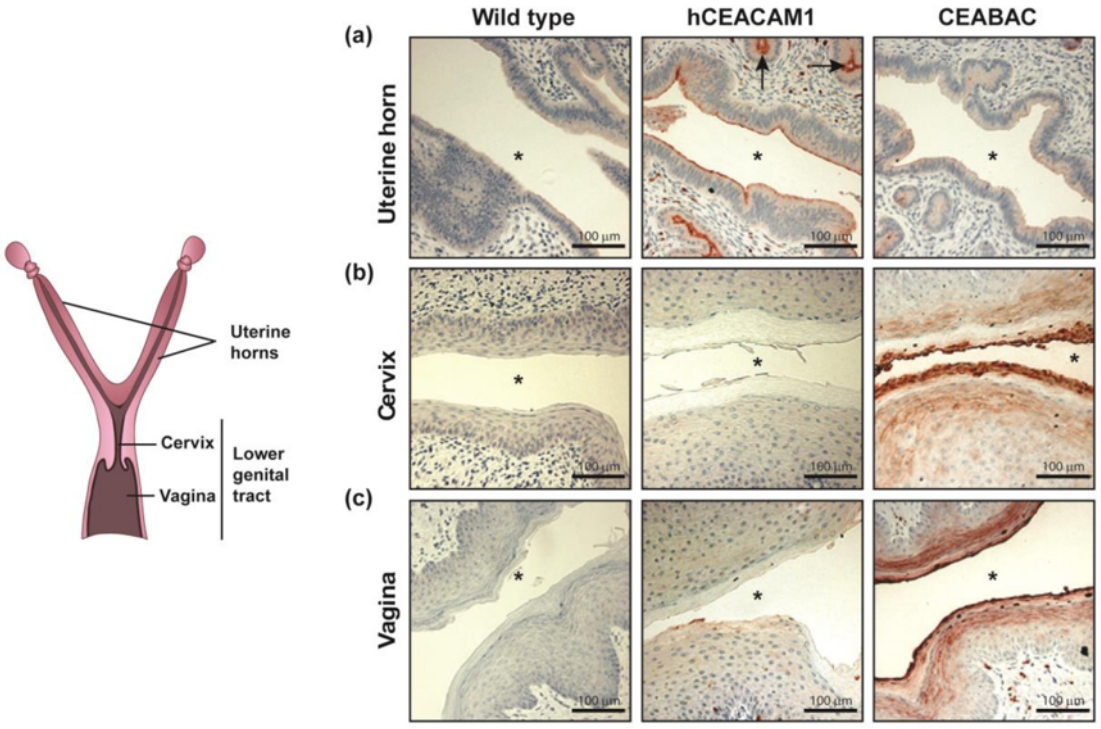


Figure 3.1: "Expression of human CEACAMs in the reproductive tract of transgenic mouse lines. Tissues from wild type and two different transgenic mouse lines were stained using a rabbit polyclonal antibody that recognizes human CEACAM1, CEACAM3, CEACAM5 and CEACAM6 (red-brown), but not any mouse CEACAM orthologues. Tissues depicted here were collected from mice at the estrus stage. Nuclei were counterstained with hematoxylin (purple). The lumen in each image is located by an asterisk; uterine glands are indicated by arrows. Images were obtained at a 20x magnification and are representative of at least 3 animals." Recreated here with permission in its entirety from (Islam et al., 2018).

The exclusive expression of hCEACAM1 and hCEACAMs 5/6 in the upper and lower FGT respectively of the transgenic mice allows for the systematic evaluation of the impact of adhesion factors on natural GC infection in the context of different reproductive cycles.

Results

Expression of human CEACAMs does not significantly impact host cytokine response to *intravaginal* infection of mice during estrus phase

In order to evaluate bacterial adhesion dependent differences in *Neisseria gonorrhoeae* infection of mice, we first examined the impact of hCEACAMs within the context of the established estrus model. Following this established protocol, we estrus phase locked 18 mice with β-estradiol, 6 each from each genotype; hCEACAM1, CEABAC2 and wild-type FvB mice. The natural microbiome of these mice was also suppressed by a poly-antimicrobial treatment (see methods for details). Once the mice were properly arrested in estrus and prepared for infection per the estrus model protocol, 3 mice from each genotype (hCEACAM1, CEABAC2 and wild-type FvB mice) were infected vaginally with the standard infectious dose of 10^7 gonococci (hCEACAM1, 3, 5, and 6 binding Opa_{57} strain of MS11 GC). The remaining 3 mice from each genotype received vaginal deposition of sterile PBS of the same volume as the infectious dose. All mice were sacrificed 6 hours after infection at which time FGT tissue and serum was collected from each mouse.

The hallmark of the estrus model is an anatomically restricted colonization of the vagina inducing proinflammatory cytokines and chemokines, with no previous description of distal tissue involvement (Packiam et al., 2010). Based on that, we examined vaginal levels of the proinflammatory cytokine Il-1α, and chemokines MIP-1α and KC. Surprisingly, we saw no significant induction of any of the three cytokines by infection let alone any genotype specific increases (Figure 3.2). As shown by figure 3.2a,

there was a weak trend towards higher Il-1α levels in the infected CEABAC2 mice as compared to the uninfected controls of the same genotype, however it was not significant by non-parametric paired *t* test (p=0.4). While slightly closer to significance, the same trend towards greater cytokine induction by infection in CEABAC2 mice was observed for the neutrophil chemokine KC (p=0.14) (Fig. 3.2b), and the monocyte chemokine MIP-1α (p=0.24) (Fig. 3.2c).

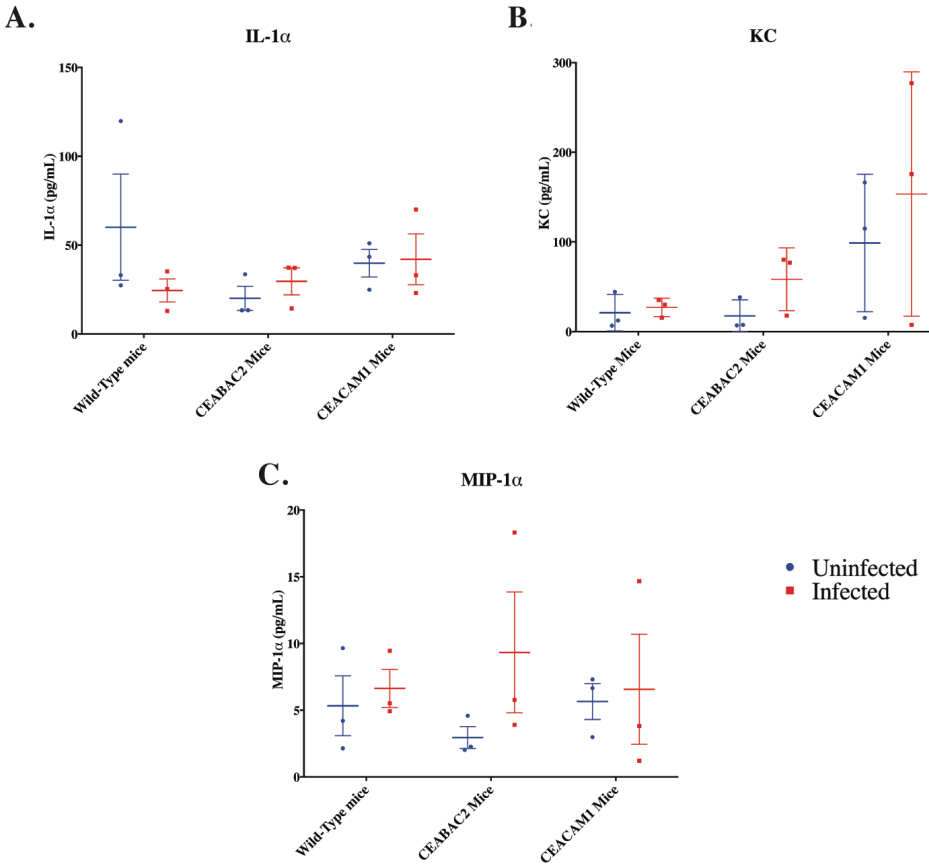


Figure 3.2: The expression of human CEACAMs does not significantly increase the murine host response to vaginal *Neisseria gonorrhoeae* infection as measured by vaginal cytokines. Levels of vaginal tissue associated IL-1α (**A**), KC (**B**), and MIP-1α (**C**) 6 hours after treatment with either 10^7 gonococci or PBS were measured by ELISA. N=3 for each treatment group. Graphs show means and standard deviation for a given group

Unsurprisingly, given the lack of a host response in the vagina, there was no evidence of upper genital tract disease in any genotype, as measured by cytokine production in isolated uterine tissue (Figure 3.3). With the exception of MIP-1α in the CEABAC2 mice (p=0.14), we did not even observe the general trend of infection induction of the cytokines that was seen in vaginal tissue.

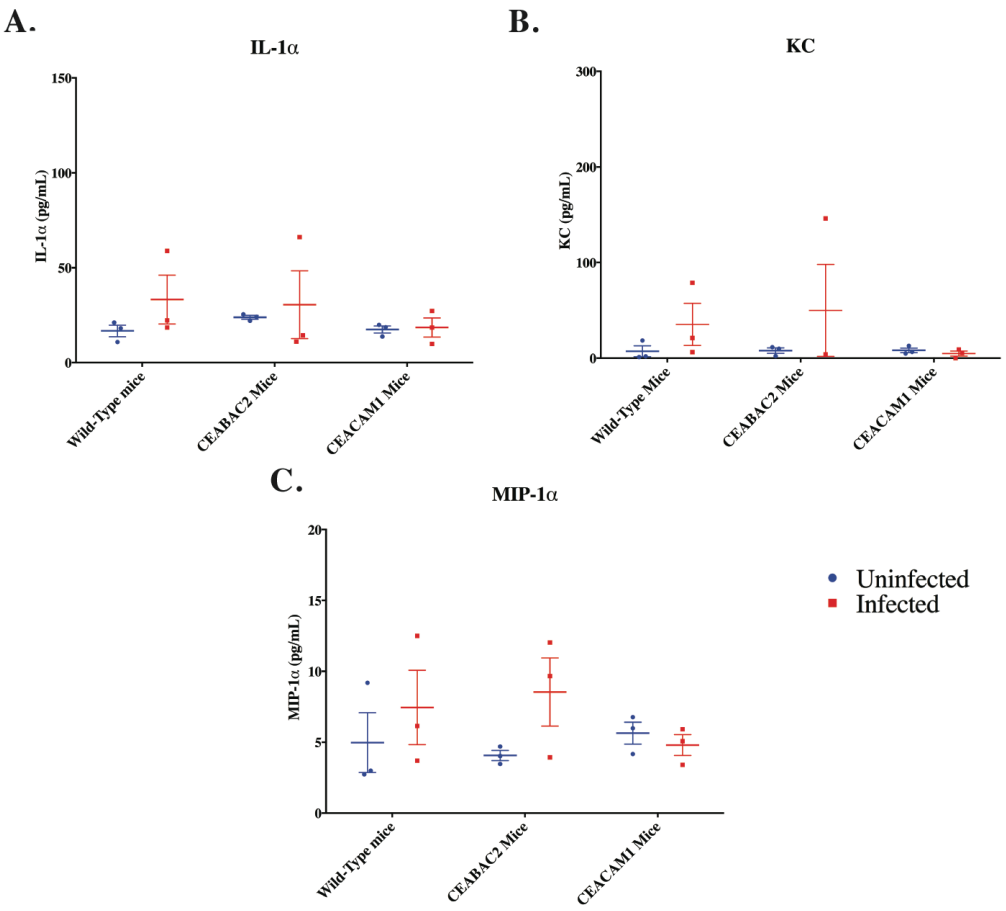


Figure 3.3: The expression of human CEACAMs does not allow for the ascension of gonococcal infection to the female murine upper genital tract as measured by uterine tissue associated cytokines. Levels of uterine tissue associated IL-1α (**A**), KC (**B**), and MIP-1α (**C**) 6 hours after treatment with either 10^7 gonococci or PBS were measured by ELISA. N=3 for each treatment group. Graphs show means and standard deviation for a given group

These results suggest that, in this specific system, the expression of human CEACAMs does not entirely abrogate murine resistance to GC infection. In fact, the poor reactivity of the wild-type FvB mice indicated that these mice appear to be fairly resistant to the induction of GC-induced inflammation. These results are not entirely surprising as the estrus model has been shown to have extreme heterogeneity between different mouse genotypes (Packiam et al., 2010), and has never been described in FvB mice before. These mice are frequently utilized in construction of transgenic lines, due to the unusually large nuclei of their ovum, allowing for easier injection of genetic material. Additionally, there are other well characterized human-restricted virulence factors that the gonococcus cannot utilize in mice (Lee and Schryvers, 1988; Ram et al., 1998b). It is also possible that this early time point may not be entirely appropriate to evaluate the response in this specific mouse line. Despite these limitations it is clear that the lower FGT hCEACAMs did not result in a distinct host response phenotype from the classic estrus model. In particular, we saw no indication of ascending infection despite, as our collaborators have shown (Islam et al., 2018), increased length of GC carriage in the murine vaginal tract in the context of hCEACAM5 expression. Since uterine GC infection in mice has not been described at all before, we wanted to investigate whether or not such an infection was even possible.

Expression of human CEACAMs results in greater cytokine induction following estrus-phase *transcervical* infection of mice

The direct infection of the murine female upper genital tract is a method that has been utilized by *Chlamydia trachomatis* researchers to model upper FGT infection that can be caused by that pathogen (Gondek et al., 2012). Here we utilized this transcervical infection method to interrogate whether a host response is even observed during murine upper FGT infection and whether or not that response is affected by the presence of hCEACAMs.

For this study, 36 mice (18 wild-type FvB, 9 CEABAC2, and 9 hCEACAM1) were prepared for infection by estrus phase locking them with β-estradiol treatments. It is important to note that these mice were not treated with antibiotics and had intact microbiomes at the site of infection, as the uterus is a sterile site and there was no worry that commensals would outcompete the pathogen and prevent infection, like with vaginal infection. Once the mice were estrus phase locked, 9 wild-type mice and all transgenic mice were infected via transcervical deposition of 10^7 gonococci directly into a uterine horn. The remaining 9 wild-type mice received transcervical deposition of sterile PBS as controls. At 6, 12 and 24 hours post infection, 3 mice from each group were sacrificed, at which point vaginal tissue, upper genital tract tissue and sera was collected separately from each mouse. To fully evaluate the immunological response of the host to uterine infection, we utilized a 20-plex mouse cytokine panel which measures quantities of a broad range of cytokines.

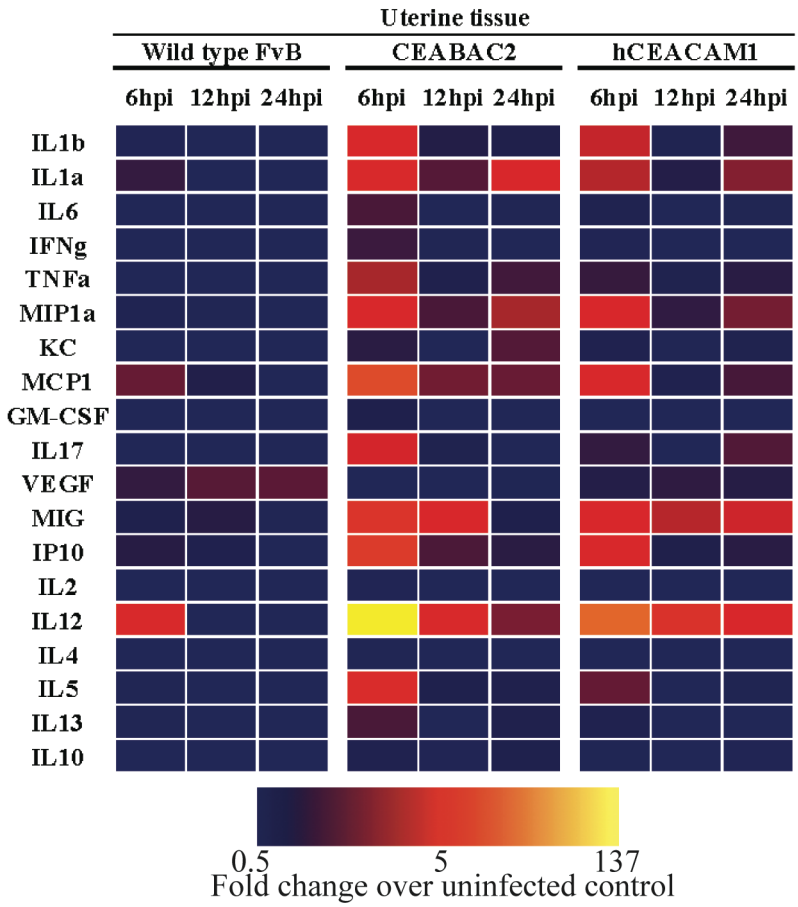


Figure 3.4: Transcervical infection of hCEACAM expressing mice results in a stronger and more diverse cytokine response in infected tissue. Uterine-tissue-associated protein levels of 20 mouse cytokines were measured by Luminex 6, 12, or 24 hours after trans-cervical treatment with 10^7 gonococci or PBS. Protein levels are presented here as fold change over uninfected controls. N=3 mice per condition/time-point, 36 total.

As shown in Figure 3.4, transcervical gonococcal infection induces a strong, genotype dependent, cytokine response in the upper genital tract (primary site of infection). Wild-type mice had a relatively modest response with strong induction of only IL-12 (11.8x fold increase over control) and only at the earliest time point. Other analytes did show induction at that same time point in the uterine tissue of infected wild type mice, however they were only very mild increases in VEGF (1.7x), IL-1a (1.7x) and

MCP1 (2.7x). The only cytokine to demonstrate prolonged induction was VEGF, which slightly increased from 1.7x at 6hpi to 2.5x fold induction at 24hpi.

Interestingly, the cytokine response in infected transgenic mice was radically different. In both transgenic mouse lines, there was robust induction over uninfected controls of several cytokines starting at 6 hours post infection (hpi) and, in the case of several analytes, persisting until final observations at 24hpi. Like with the wild-type mice, the greatest difference was seen in IL-12 levels. The induction of this classic Th1 associated cytokine, was much more potent in the transgenic mice with CEABAC2 mice exhibiting an average fold induction of 137.3x over uninfected controls. The hCEACAM1 mice demonstrated ~62x greater IL-12 levels than that found in uninfected tissue. In addition to demonstrating a greater magnitude cytokine response to GC infection, the transgenic mice responded with a more complex cytokine profile. In particular there was a strong chemokine response. Both genotypes saw strong induction of the monocyte chemokines MIP-1α, MCP-1, IP-10 and the T-cell chemokine MIG. Once again, the response was slightly greater in the CEABAC2 mice than hCEACAM1 mice. This is particularly interesting since, the CEABAC2 mice do not express hCEACAMs in the uterus so increased bacterial adhesion is not facilitating the greater response. Instead, it is possible that the hCEACAM3 decoy receptor expressed on the neutrophils of CEABAC2 mice might result in greater engagement and activation of the innate immune system during infection. This is supported by the greater induction of IL-1β in CEABAC2 mice, which has been shown to be induced by GC engagement of hCEACAM3 on neutrophils (Sintsova et al., 2014; Islam et al., 2018). In addition to IL-

1β, CEABAC2 mice showed unique or particular induction of IL-1α, TNF-α, IL-17, and IL-5. This complex cytokine response is suggestive of a highly inflammatory state, indicated by Il-1α/β, TNF-α, and to a point Il-17. This Th17 cytokine has a pleomorphic effect that can facilitate both (frequently detrimental) inflammation and mucosal surface immunity (Guglani and Khader, 2010). While the cytokine response of CEABAC2 mice to transcervical GC infection suggest a greater immune engagement and response, they also demonstrated relatively quick (compared to hCEACAM1 mice) resolution of that response. Both Il-12 and MIG levels remained highly elevated in hCEACAM1 mice at the 24hpi time point, while CEABAC2 mice had returned almost to baseline levels. This prolongation of the host response could be the result of greater retention of bacteria due to the presence of adhesion targets. In fact, our collaborators have shown greater gonococcal binding, retention and uterine tissue invasion following transcervical infection of hCEACAM1 mice (Islam et al., 2018).

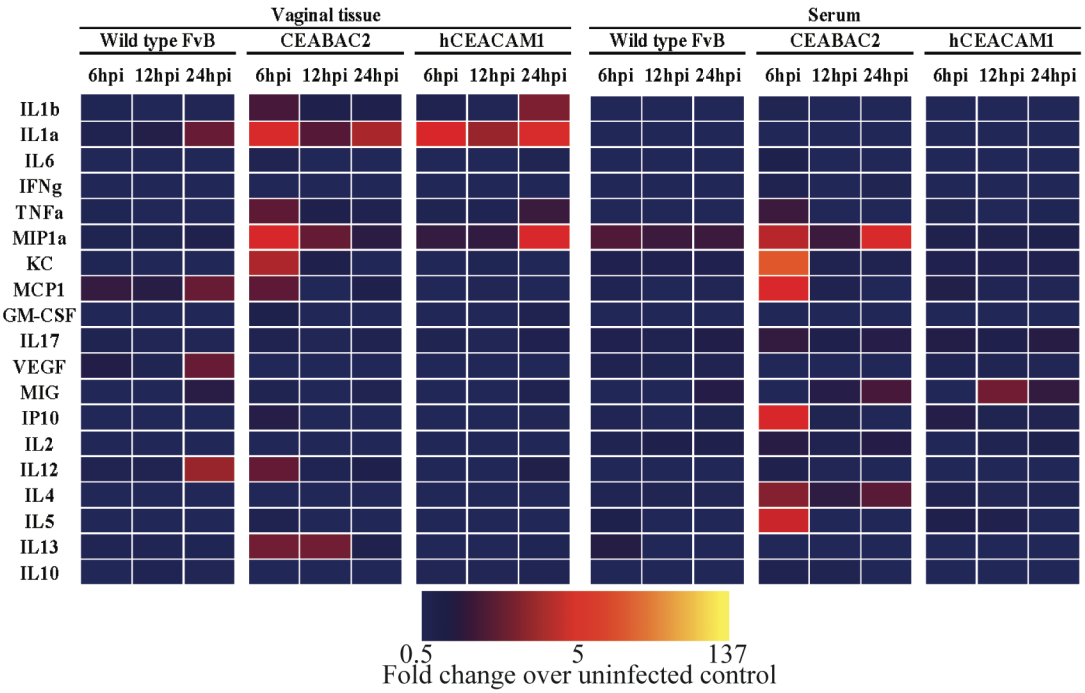


Figure 3.5: A host response to GC, distal from the site of primary infection, is induced in mice that express hCEACAMs. Vaginal-tissue and sera associated protein levels of 20 mouse cytokines were measured by Luminex 6, 12, or 24 hours after trans-cervical treatment with 10^7 gonococci or PBS. Protein levels are presented here as fold change over uninfected controls. N=3 mice per condition/time-point, 36

As shown by figure 3.5, the cytokine response, and likely the infection, was relatively restricted to the upper genital tract. There was a significant induction of vaginal Il-1α and MIP-1α in the two transgenic mouse groups. This response was sustained in the hCEACAM1 mice which may be indicative of longer retention in the upper FGT and therefore prolonged bacterial shedding into the lower FGT. Interestingly, there was a measurable systemic response in CEABAC2 mice with particular induction of the neutrophil chemokine KC. Once again, this is likely due to neutrophil activation through hCEACAM3 particularly since this reaction was not described by our collaborators in hCEACAM5 expressing mice upon transcervical infection (Islam et al., 2018).

These results demonstrate that transcervical deposition of *Neisseria gonorrhoeae* is able to induce a robust cytokine response within the uterus itself. This suggests that the failure to observe upper genital tract disease in the classic estrus mouse model is likely due to a failure of the infection to ascend into the uterus rather than an inherent resistance to upper FGT disease. In addition to showing that upper FGT infection produces a robust host response, these results show that expression of hCEACAMs significantly impacts the process of GC infection in mice. Expression of the target of uterine bacterial adhesion, hCEACAM1, resulted in a more robust cytokine response in infected tissue, than that in wild-type mice. That response showed a strong recruitment of immune cells, particularly monocytes and T-cells, to the site of infection, an induction of the proinflammatory cytokines IL-1α and β, and finally the powerful induction of the T-cell regulating cytokine IL-12. Most interesting was the persistence of IL-12 and the T-cell chemokine MIG as it suggests a prolonged interaction between the bacteria and the immune system that was hypothesized in the context of upper FGT adhesion expression. Similarly, it was shown that in the context of hCEACAMs 3, 5, and 6 upper FGT infection induced even stronger cytokine induction and induced significant levels of the potent inflammatory cytokines TNF-α and Il-17. These results, in the context of our collaborators findings of mild pathology in hCEACAM5 expressing mice (Islam et al., 2018), suggest hCEACAM3 expression in mice allows for greater immune system engagement with and response to the pathogen. The ability to measure the host response to uterine infection of transgenic mice, in the lower FGT and sera further suggests hCEACAM expression significantly increases the ability of GC to interact with the host

immune system and induce inflammation and immune cell movement into infected tissue, both of which are hallmarks of active gonorrhea in humans.

Transcervical infection of female mice during diestrus results in a more robust cytokine response than during estrus

It has been previously discussed that the natural female reproductive cycle seems to have a significant impact on the course of natural gonococcal infection in humans. The relative permissiveness or resistance of the murine lower FGT to gonococcal infection depending on the reproductive phase at the time of infection suggests that this process has a significant impact on murine GC infection as well. While the lower FGT of mice has been repeatedly characterized as being resistant to GC infection during diestrus, no studies have attempted to characterize uterine infection during diestrus.

To evaluate estrous phase dependent differences in the host response to murine upper FGT *Neisseria gonorrhoeae* infection, 24 wild type mice were evenly separated into estrus and diestrus phase. Like with previous transcervical infection studies, these mice were not treated with any antibiotics. For this study, in order to evaluate the effect of the natural cycle, the mice were not treated with exogenous hormones either. The mice were instead separated into their natural reproductive-cycle phase based on vaginal epithelial cell morphology (see methods). Once an even population of mice were divided into their phases, 8 mice from each phase were infected transcervically as previously described and the remaining 4 were treated with transcervical PBS. At 6hpi, 3 uninfected estrus phase mice (1 mouse had to be sacrificed early due to injury and was excluded

from the study), 4 infected estrus phase mice, 4 uninfected diestrus phase mice, and 4 infected diestrus phase mice were sacrificed and upper and lower genital tract tissue and sera were collected separately from each mouse. The remaining 4 infected mice from each phase were sacrificed and had tissue collected at 18hpi. Once again, the host response to infection was evaluated by the induction of a wide range of cytokines in primary infected tissue (the uterus), in the lower FGT, and finally systemically as compared to uninfected control tissue.

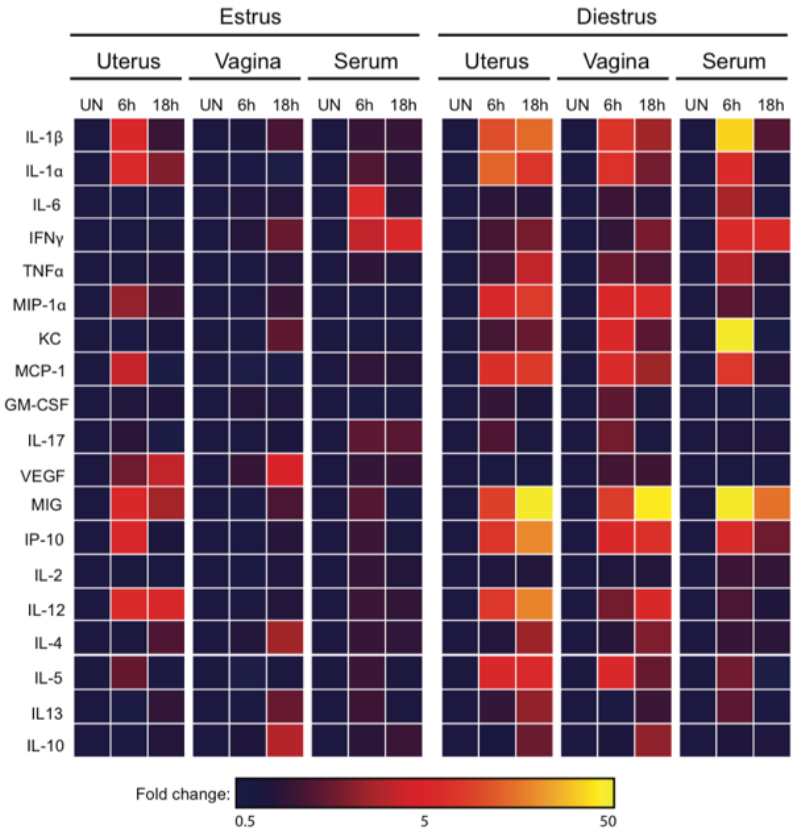


Figure 3.6: Transcervical infection in diestrus is more inflammatory as measured by cytokine induction. Protein levels of 20 murine cytokines in uterine tissue, vaginal tissue and sera were measured by Luminex assay. Tissues were collected from wild-type mice either at time 0, 6 hours or 18 hours after treatment with either 10^7 gonococci or PBS (N=4 per condition except N=3 for PBS Estrus). Expression levels of each cytokine are presented as fold change over uninfected controls

Similar to what was observed in the studies of transcervical infection in estrus phase, the mice in this study that were infected in estrus demonstrated a mild induction of a range of cytokines with the greatest inductions being in IL-12 and IL-1α and IL-1β. While the heatmap appears to show greater induction of these and other cytokines in the estrus mice than what was previously observed, this is an artifact of the narrower induction range and subsequent heatmap gradient (0.5-50 here as compared to 0.5-137 above). Likewise, the vaginal and serum cytokines, shown here to be induced in transcervical estrus infection, largely reflect what was previously described.

In contrast, transcervical infection of mice in diestrus induced an extremely powerful host response that extended beyond the site of infection and involved the vaginal tissue and sera as well. The cytokine profile was essentially identical to that of estrus infection but the diestrus tissues exhibited greater magnitude induction, as well as longer duration increases. A few notable cytokines were uniquely or disproportionately induced in diestrus infection than estrus. The two most interesting of these cytokines are KC and TNFα. KC is the primary neutrophil chemoattractant in murine physiology (Lee et al., 1995). The recruitment to and activation of neutrophils at the site of infection is one of the most striking clinical characteristics of human gonorrhea. The fact that this chemokine is so potently induced both locally in the uterus and systemically suggests an induction of a purulent inflammation that may be reminiscent of active human infection. The particular induction of TNFα further supports the idea of a particularly potent inflammatory response when transcervical infection occurs in diestrus.

In addition to these cytokine differences, our collaborators described increased bacterial penetration into the uterine tissue, extreme inflammation of the uterine horns, and clinical signs of distress during transcervical diestrus infection (Islam et al., 2016). These observations of transcervical infection of diestrus mice describe a clinical and pathological picture that is reminiscent of human PID, particularly when compared to the relatively mild response of estrus mice to transcervical infection. This potential murine PID model could be invaluable in the development of next generation anti-gonococcal therapeutics however it must be more thoroughly characterized.

Transcervical infection of female mice during diestrus more profoundly induces immunological transcriptional profiles

To examine the transcriptional differences in host response underlying the strikingly distinct phenotypes induced by transcervical GC infection in the estrus and diestrus phases, we compared transcriptional profiles from uterine tissue extracted from mice in 4 distinct experimental groups; transcervical PBS treated mice during diestrus phase (4 mice), transcervical GC infected mice during diestrus phase (4 mice), transcervical PBS treated mice during estrus phase (3 mice), and transcervical GC infected mice during estrus phase (4 mice). Tissue collected 6 hours post infection was examined as it appeared to be closest to the peak of the previously described host response.

To best examine the impact of our two variables (reproductive-cycle phase and infection state) and the interaction of the two on transcription, we modeled gene

expression as a linear function of reproductive-cycle phase, infection state, and the interaction between phase and infection state (phase:infection). For each model, moderated *t* tests were performed on the corresponding coefficient of the linear model to obtain a *t* statistic and *p* value for each gene. In order to account for multiple comparison testing error, Benjamini-Hochberg false discovery rate (FDR) correction was then applied to obtain corrected *p* values (*q* values) after removing genes that were not expressed above the median value of at least one array. This analysis identifies genes whose expression is significantly impacted by one of our variables, after correcting for the effect of the other; or in the case of phase:infection interaction, the *t* statistic generated measured the significance of a combined effect of the variables on a gene's expression.

Neisseria gonorrhoeae infection induces immune gene expression regardless of hormone cycle phase

Using the previously described linear modeling approach, expression was modeled as a function of infection state. We identified a large population of genes whose expression was significantly associated with infection state after correcting for reproductive cycle effects (516 genes with FDR $q < 0.1$). The biological context of those 516 genes was provided by pre-ranked Gene Set Enrichment Analysis (GSEA) performed using the infection *t* statistic (Subramanian et al., 2005; Subramanian et al., 2007), which identified 449 gene sets that showed significant (FDR $q < 0.25$) coordinate expressional regulation with respect to infection. Those gene sets that demonstrated the most significant positive coordination of expression (or upregulation in infected compared to

uninfected tissues) (FDR $q < 0.001$) were almost exclusively related to the host immune response, including the gene sets "chemokine receptors bind chemokines" (Reactome; R-HSA-380108) and “cytokine cytokine-receptor interaction” (KEGG; HSA04060).

The genes from each gene set that most contributed to the significance of the set’s infection effect, referred to as the leading-edge genes, exhibit an interesting expression pattern (Figure 3.7). The cytokine gene sets demonstrate clear phase-independent induction with expression in infected tissues being appreciably higher than their uninfected phase-matched controls. Several of the cytokines and chemokines found at the leading edge of these sets are suggestive of gonorrhea’s characteristic recruitment of leukocytes to infected tissues. These genes include the neutrophil chemokines *Cxcl5*, *Ccl4* and *Cxcl1*, as well as the T cell chemokines *Ccl5*, *Cxcl10* and *Ccl17*. Gonorrhea’s strong inflammatory reaction was also reflected in this phase-independent anti-GC response with classic proinflammatory mediators like *Il1a* and *Il1b*, *Ltb* (Lymphotoxin Beta), and *Tnf* (TNF-α) found among the leading-edge genes. The presence of immune function gene sets, driven by proinflammatory cytokine and chemokine expression induction, in the infection effect GSEA suggests an anti-gonococcal response common to all reproductive phases characterized by local inflammation and immune cell invasion. Despite the apparent universality of this response to GC infection, our previous descriptions of profound phenotypic differences, in these same pathways, between infection during estrus and diestrus phases suggests a more complex process at work.

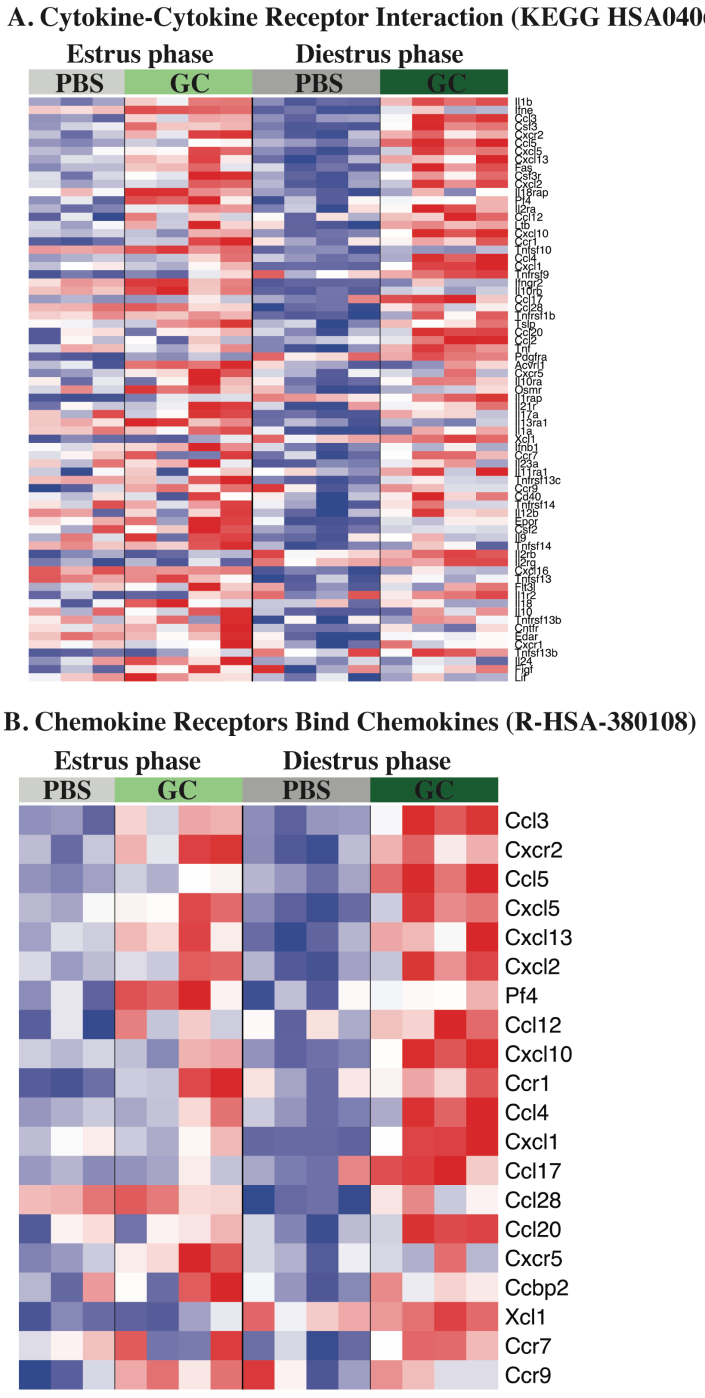


Figure 3.7: Leading edge genes from GSEA identified phase effect cytokine gene sets show phase-independent induction but phase-dependent induction magnitude. The infection dependent host response was evaluated by microarray analysis of mRNA extracted from infected uterine tissue collected 6 hours after initial treatment with 10^7 gonococci or PBS. Expression levels of leading edge genes from for top gene sets identified by infection-effect GSEA are presented; "Cytokine-Cytokine Receptor Interaction" (KEGG HSA04060) (**A.**) and "Chemokine Receptors Bind Chemokines" (R-HSA-380108) (**B.**). For each gene, expression values (log2(expression)) are normalized to a mean of zero and standard deviation of one (z-normalized) for visual representation so that red and blue indicate z-scores of ≥ 2 or ≤ -2, respectively, and white indicates a z-score of 0 (row-wise mean). Genes are presented in descending significance of infection effect *t* statistic (top to bottom). (N=4 per condition except N=3 for PBS Estrus)

These differences in infection phenotype may be at least partially due to differential magnitudes of activation of this common anti-gonococcal response. Evidence of this can be found in the same genes highlighted as the hallmarks of the general anti-

GC response, as they display unequal induction by infection. This is particularly striking with the neutrophil chemokines. For example, *Cxcl1*, while upregulated in all infected tissues, expression in diestrus tissue is lower at baseline and greater in infection as compared to estrus. This larger magnitude in diestrus is found generally in both the chemokine and cytokine gene sets (Figure 3.7). These observations indicate that unequal activation of a common anti-gonococcal host response, characterized by inflammatory and cell recruitment processes, at least partially is responsible for phenotypic differences between infected estrus and diestrus tissue.

Genes exhibiting significant reproductive-cycle-dependent infection responses separate into distinct expression patterns

To evaluate reproductive-phase-dependent transcriptional differences in the host response to transcervical infection, we applied the same analytical approach as employed above for phase and infection effect. For this analysis however, the modeled linear function was expression as a function of the interaction between reproductive-cycle phase and infection state (phase:infection). Applying moderated *t* tests on the resulting coefficient of the linear model we were able to identify genes that had different expressional changes between the two reproductive-cycle phases, in response to infection. This analysis identified 416 genes (FDR $q < 0.25$) subject to significant phase:infection effect, which clustered into 6 distinct patterns of expression (Figure 3.8a).

Genes with increased expression only in estrus-phase infection are found in cluster 1 and represent diverse biological functions (Figure 3.8b). Of the 64 genes in

cluster 1, only a few are potentially immunologically significant do not strongly suggest specific estrus-infection immune processes. The low affinity IL17 receptor, encoded for by *IL17ra*, is found in cluster 1. Since this cytokine has demonstrated a wide variety of functions and this specific receptor itself has been tied to cutaneous homeostasis, mucosal immune responses, and potentiation of antibody driven autoimmunity, it is difficult to interpret its presence in this cluster without additional members of any of those biological pathways (Scurlock et al., 2011; Ding et al., 2013; Ramani et al., 2014; Lombard et al., 2016; Floudas et al., 2017). Similar ambiguity of function surrounds the other immune genes found in cluster 1. The activating receptor encoded for by *Cd300lb* can be found both in myeloid cell membranes or secreted, serving two distinct functions(Yamanishi et al., 2012; Borrego, 2013). While the lack of corroborating genes in cluster 1 obscures the role of this gene in the less inflammatory phenotype of estrus GC colonization, the literature may explain the increased *Cd300lb* expression as the result of increased neutrophil expression and secretion of the receptor in response to LPS by the previously described larger neutrophil population in the murine uterus during estrus (Yamanishi et al., 2012). Perhaps the most intriguing immune gene found in cluster 1 is *Trem2*. This gene encodes for an anti-inflammatory receptor expressed on innate immune cells that binds to and response specifically to LPS (Gawish et al., 2015). Like with the previously discussed genes, it is hard to comment on any broader biological impact of the increased expression of *Trem2* without coincident increases in known co-mediators of a given pathway, however the strong anti-inflammatory function of the receptor could contribute to the dampened inflammatory response observed in GC infection during estrus.

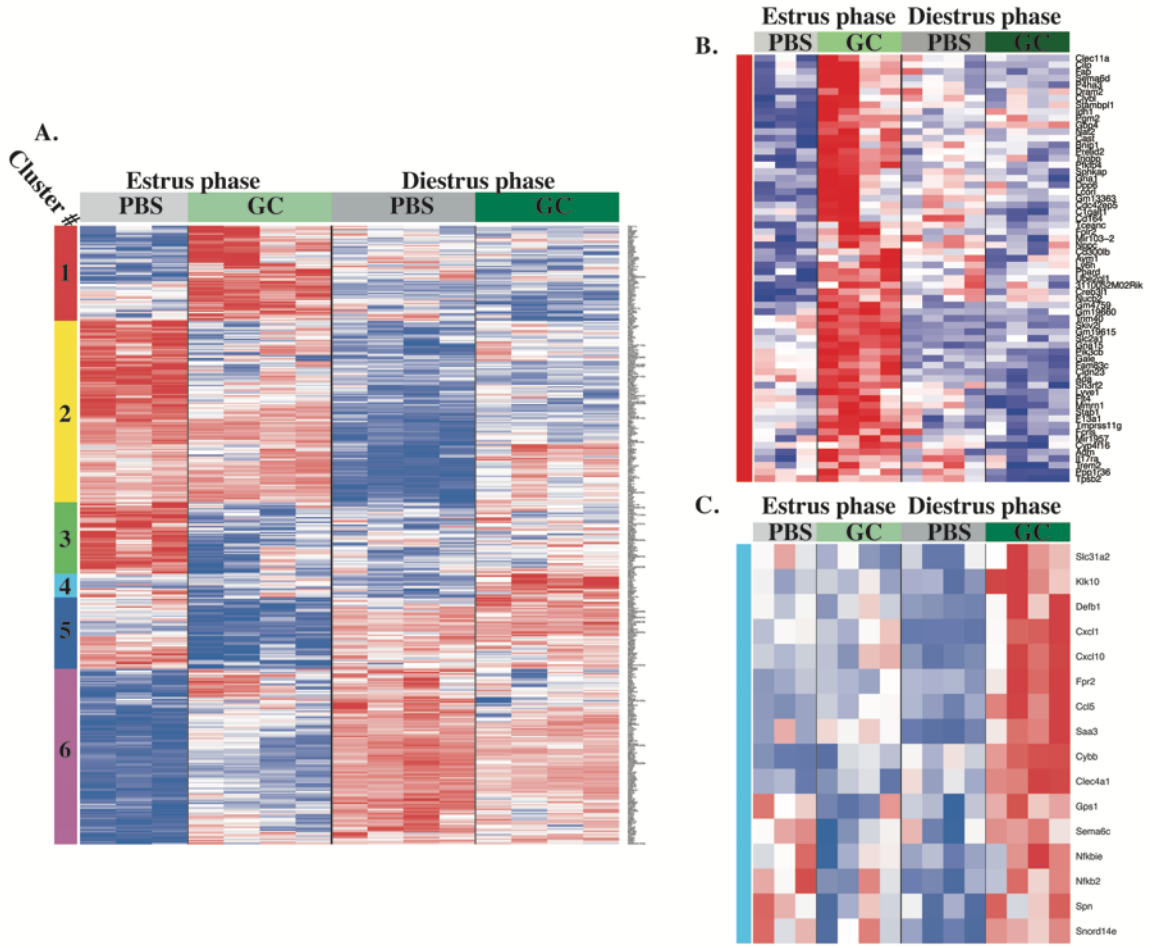


Figure 3.8: Genes with significant phase:infection interaction effect cluster into distinct expression patterns. Reproductive phase dependent elements of the anti-GC host response were evaluated through identification of phase:infection effected genes within microarray analysis of mRNA extracted from infected uterine tissue collected 6 hours after initial treatment with 10^7 gonococci or PBS. Expression levels of (**A**) All 416 genes with phase:infection FDR $q < 0.25$ were clustered based on their relative expression pattern across all samples. Clusters are indicated by colored sidebar and number. Rows represent genes, with log2(expression) values z-normalized (to a mean of zero and a standard deviation of one) across all samples. Colors are scaled so that red and blue indicate z-scores of ≥ 2 or ≤ -2, respectively, and white indicates a z-score of 0 (row-wise mean). (**B**) Enlarged view of Cluster 1, comprised of genes induced specifically in estrus phase. (**C**) Enlarged view of Cluster 2, comprised of genes induced specifically in diestrus phase. N=4 for all conditions except N=3 for PBS Estrus.

The remaining genes in the cluster are associated with either non-immunologically relevant pathways or broad non-specific functions with minor (if any)

immunological implications. The latter is best exemplified by Pik3cb, which encodes an isoform of a regulatory protein in the phosphatidylinositol signaling system, an expansive system with some components active in immune processes. Other processes represented in cluster 1 include metabolism (e.g. Pfkfb4) and extracellular structure (e.g. P4ha3) which may reflect further increases in the baseline biologic functions in the estrus uterine tissue (buildup and maintenance of the thick uterine lining) within the context of mild physiologic stress caused by bacterial colonization.

Cluster 4 encompasses transcripts upregulated only during diestrus-phase infection. In contrast to the estrus-restricted responses of cluster 1, this cluster is comprised almost entirely of immunologically active genes (Figure 3.8c). These include the previously discussed chemokines Cxcl1, Cxcl10, and Ccl5; the chemoattractant receptor Fpr2, which has an identified role in host response to some bacterial infections (Kretschmer et al., 2010); the endogenous antimicrobials beta-defensin 1 (Defb1) and reactive-oxygen-species (ROS) producing enzyme NOX2 (Cybb); and major regulators of general immune activation including a component of the classic pro-inflammatory transcription factor, Nuclear Factor kappa-B (Nfkb2). These findings lend transcriptomic context for the stark phenotypic differences observed in *Neisseria gonorrhoeae* transcervically infected mice during the diestrus and estrus phases of the reproductive cycle. The remarkable absence of immune genes induced exclusively in estrus infection suggests a lack of a unique anti-bacterial host response beyond the previously discussed common anti-gonococcal response. This stands in stark contrast to the diestrus infection specific induction of a cohesive set of genes that clearly indicate induction of specific

host response pathways including immune cell effector function and anti-bacterial action, in addition to the cell recruitment and inflammation of the common anti-gonococcal response.

Infection in diestrus phase induces members of immunologically relevant gene sets

In order to fully evaluate differences in biological processes associated with GC infection in different reproductive-cycle phases, we once again performed a pre-ranked GSEA, this time using the phase:infection interaction *t* statistic (Subramanian et al., 2005; Subramanian et al., 2007). This GSEA identified 70 gene sets with significant (FDR $q < 0.25$) coordinate expression of genes displaying a pattern of greater upregulation (or less downregulation) in diestrus phase infection than in estrus phase infection (Table 3.1). Interestingly the significant gene sets included not only those suggestive of the inflammation and cell recruitment focused common anti-gonococcal response, but additional sets suggesting greater type I interferon signaling (Table 3.1 set: "Interferon alpha beta signaling"), pattern recognition receptor (PRRs) activity (Table 3.1 sets: "TLR signaling pathway", "NLR signaling pathway", "Detection of a stimulus"), and immune cell activation/function (Table 3.1 sets: "CD40 pathway", "NFKB pathway", Myeloid cell differentiation", "Leukocyte differentiation", "Immune Effector Process") in diestrus infection. The top sets in the GSEA indicated particular activation of interferon and chemokine activity.

Group	Gene Set Name	Normalized Enrichment Score (NES)	Nominal p value	FDR q value
Reactome pathway	REACTOME_INTERFERON_ALPHA_BETA_SIGNALING	2.50	0.0000	***0.0000***
GO Molecular Function	GO CHEMOKINE_ACTIVITY	2.34	0.0000	***0.0000***
GO Molecular Function	GO CHEMOKINE_RECEPTOR_BINDING	2.19	0.0000	***0.0006***
GO Molecular Function	GO CALMODULIN_BINDING	1.99	0.0000	***0.0454***
GO Biological Process	GO RESPONSE_TO_OTHER_ORGANISM	1.99	0.0000	***0.0371***
GO Biological Process	GO MYELOID_CELL_DIFFERENTIATION	1.97	0.0000	***0.0401***
KEGG pathway	KEGG_OLFACTORY_TRANSDUCTION	1.95	0.0000	***0.0426***
GO Molecular Function	GO G_PROTEIN_COUPLED_RECEPTOR_BINDING	1.95	0.0000	***0.0376***
BioCarta pathway	BIOCARTA_NFKB_PATHWAY	1.95	0.0000	***0.0361***
GO Molecular Function	GO ANION_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	1.93	0.0000	***0.0399***
BioCarta pathway	BIOCARTA_TNFR2_PATHWAY	1.93	0.0000	***0.0383***
KEGG pathway	KEGG_TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY	1.91	0.0018	***0.0470***
Reactome pathway	REACTOME_CGMP_EFFECTS	1.90	0.0019	***0.0491***
GO Biological Process	RESPONSE_TO_VIRUS	1.87	0.0017	***0.0643***
KEGG pathway	KEGG_CYTOSOLIC_DNA_SENSING_PATHWAY	1.86	0.0000	***0.0706***
KEGG pathway	KEGG_TYPE_I_DIABETES_MELLITUS	1.85	0.0017	***0.0760***
KEGG pathway	KEGG_NOD_LIKE_RECEPTOR_SIGNALING_PATHWAY	1.84	0.0018	***0.0750***
Reactome pathway	REACTOME_OLFACTORY_SIGNALING_PATHWAY	1.84	0.0000	***0.0724***
Reactome pathway	REACTOME_TRAF6_MEDIATED_IRF7_ACTIVATION	1.82	0.0040	***0.0869***
GO Biological Process	GO DETECTION_OF_STIMULUS	1.81	0.0000	***0.0917***
GO Molecular Function	GO CYTOKINE_ACTIVITY	1.81	0.0000	***0.0948***
TF motif	GOGTTRYCATRR_UNKNOWN	1.80	0.0000	***0.0974***
BioCarta pathway	BIOCARTA_TALL1_PATHWAY	1.79	0.0093	***0.0985***
Reactome pathway	REACTOME_INTERFERON_SIGNALING	1.79	0.0000	***0.0971***
KEGG pathway	KEGG_RIG_I_LIKE_RECEPTOR_SIGNALING_PATHWAY	1.79	0.0000	***0.0954***
Reactome pathway	REACTOME_NUCLEOTIDE_BINDING_DOMAIN_LEUCINE_RICH_REPEAT_CONTAINING_RECEPTOR_NLR_SIGNALING_PATHWAYS	1.78	0.0037	***0.0968***
TF motif	STTTCRNTTT_V$IRF_Q6	1.78	0.0000	***0.0946***
Reactome pathway	REACTOME_RIG_I_MDA5_MEDIATED_INDUCTION_OF_IFN_ALPHA_BETA_PATHWAYS	1.78	0.0000	***0.0931***
GO Biological Process	DEFENSE_RESPONSE	1.78	0.0000	***0.0903***
Reactome pathway	REACTOME_DEGRADATION_OF_THE_EXTRACELLULAR_MATRIX	1.77	0.0035	***0.0937***
Reactome pathway	REACTOME_NITRIC_OXIDE_STIMULATES_GUANYLATE_CYCLASE	1.77	0.0075	***0.0959***
Reactome pathway	REACTOME_HS_GAG_BIOSYNTHESIS	1.76	0.0074	***0.0983***
TF motif	GGGNNTTTCC_V$NFKB_Q6_01	1.73	0.0000	***0.1324***
GO Biological Process	INFLAMMATORY_RESPONSE	1.72	0.0000	***0.1410***
BioCarta pathway	BIOCARTA_IL1R_PATHWAY	1.72	0.0093	***0.1382***
BioCarta pathway	BIOCARTA_CD40_PATHWAY	1.72	0.0074	***0.1384***
Reactome pathway	REACTOME_INTERFERON_GAMMA_SIGNALING	1.71	0.0035	***0.1348***
KEGG pathway	KEGG_ARRHYTHMOGENIC_RIGHT_VENTRICULAR_CARDIOMYOPATHY_ARVC	1.71	0.0000	***0.1428***
GO Cellular Component	NUCLEAR_CHROMOSOME	1.70	0.0074	***0.1506***
GO Biological Process	LEUKOCYTE_DIFFERENTIATION	1.70	0.0019	***0.1475***
GO Biological Process	RESPONSE_TO_BIOTIC_STIMULUS	1.69	0.0000	***0.1532***
GO Biological Process	IMMUNE_SYSTEM_DEVELOPMENT	1.69	0.0035	***0.1545***
GO Biological Process	IMMUNE_EFFECTOR_PROCESS	1.68	0.0090	***0.1569***
GO Biological Process	CHROMATIN_MODIFICATION	1.68	0.0037	***0.1599***
BioCarta pathway	BIOCARTA_TID_PATHWAY	1.68	0.0131	***0.1589***
GO Molecular Function	CHLORIDE_CHANNEL_ACTIVITY	1.66	0.0074	***0.1754***
GO Molecular Function	ANION_CHANNEL_ACTIVITY	1.66	0.0114	***0.1777***
Reactome pathway	REACTOME_PRE_NOTCH_TRANSCRIPTION_AND_TRANSLATION	1.66	0.0162	***0.1773***
GO Biological Process	AMINO_SUGAR_METABOLIC_PROCESS	1.65	0.0074	***0.1817***
GO Biological Process	REGULATION_OF_MYELOID_CELL_DIFFERENTIATION	1.64	0.0097	***0.2036***
KEGG pathway	KEGG_DILATED_CARDIOMYOPATHY	1.63	0.0000	***0.2021***
TF motif	V$NFKAPPAB_01	1.63	0.0000	***0.2021***
GO Biological Process	HISTONE_MODIFICATION	1.63	0.0202	***0.2014***
Reactome pathway	REACTOME_DNA_STRAND_ELONGATION	1.63	0.0198	***0.2026***
GO Biological Process	HEMOPOIETIC_OR_LYMPHOID_ORGAN_DEVELOPMENT	1.63	0.0000	***0.2026***
GO Biological Process	HEMOPOIESIS	1.62	0.0000	***0.2039***
GO Biological Process	COVALENT_CHROMATIN_MODIFICATION	1.62	0.0215	***0.2007***
BioCarta pathway	BIOCARTA_NTHI_PATHWAY	1.62	0.0208	***0.2090***
Reactome pathway	REACTOME_TRAF6_MEDIATED_NFKB_ACTIVATION	1.61	0.0202	***0.2125***
GO Biological Process	ESTABLISHMENT_AND_OR_MAINTENANCE_OF_CHROMATIN_ARCHITECTURE	1.61	0.0069	***0.2134***
GO Biological Process	DETECTION_OF_EXTERNAL_STIMULUS	1.61	0.0254	***0.2104***
GO Cellular Component	EXTRACELLULAR_SPACE	1.60	0.0000	***0.2293***
GO Biological Process	RESPONSE_TO_BACTERIUM	1.59	0.0326	***0.2339***
Reactome pathway	REACTOME_HEPARAN_SULFATE_HEPARIN_HS_GAG_METABOLISM	1.59	0.0139	***0.2328***
GO Molecular Function	SYMPORTER_ACTIVITY	1.59	0.0204	***0.2382***
TF motif	V$CREL_01	1.58	0.0000	***0.2389***
GO Biological Process	RESPONSE_TO_UV	1.58	0.0254	***0.2365***
BioCarta pathway	BIOCARTA_STRESS_PATHWAY	1.58	0.0190	***0.2343***
KEGG pathway	KEGG_ALLOGRAFT_REJECTION	1.58	0.0259	***0.2458***
GO Biological Process	MORPHOGENESIS_OF_AN_EPITHELIUM	1.58	0.0287	***0.2423***

Table 3.2: Gene sets significantly associated with positive phase:infection *t* statistics. The differential activity of biologic systems within the phase:infection interaction effected genes was evaluated by GSEA. Seventy gene sets were identified as significantly (FDR $q < 0.25$) coordinately up-regulated to a greater degree (or down-regulated to a lesser degree) during infection in diestrus phase than in estrus phase. Gene sets are ranked in descending order by Normalized Enrichment score, and are labeled according to the MSigDB sub-collection to which they belong.

To better evaluate gene expression patterns in top GSEA sets, we examined their leading-edge genes (Figure 3.9). The leading-edge genes of the chemokine activity gene set (GO term GO:0008009) (Figure 3.9a) repeat the same pattern that has been seen throughout the analysis: induction by infection over low levels of expression in uninfected tissue that is much greater in magnitude in diestrus phase. In fact, several of the cytokines seen here are the same neutrophil chemokines (Cxcl5, Ccl4, Cxcl1), T-cell chemokines (Cccl5, Cxcl10, Ccl17) that were highlighted in the infection effect analysis. This inclusion of similar gene sets containing the same genes reinforces the greater induction of the common anti-gonococcal response in diestrus. The chemokine with one of the greatest differences in estrus and diestrus infection dependent induction was *Ccl20*. This lymphocyte chemokine is active in the mucosal adaptive immune response in the gastrointestinal tract particularly in response to bacterial infection (Cook et al., 2000; Yamazaki et al., 2008) (Hoover et al., 2002; Ravindran et al., 2007). While we could not find any description of CCL20 induction by GC specifically, there is evidence that production of this chemokine can be induced by bacterial products (Radtke et al., 2012) and is suppressed by estrogen (Haddad and Wira, 2014), which together would explain its particularly potent induction in diestrus infection.

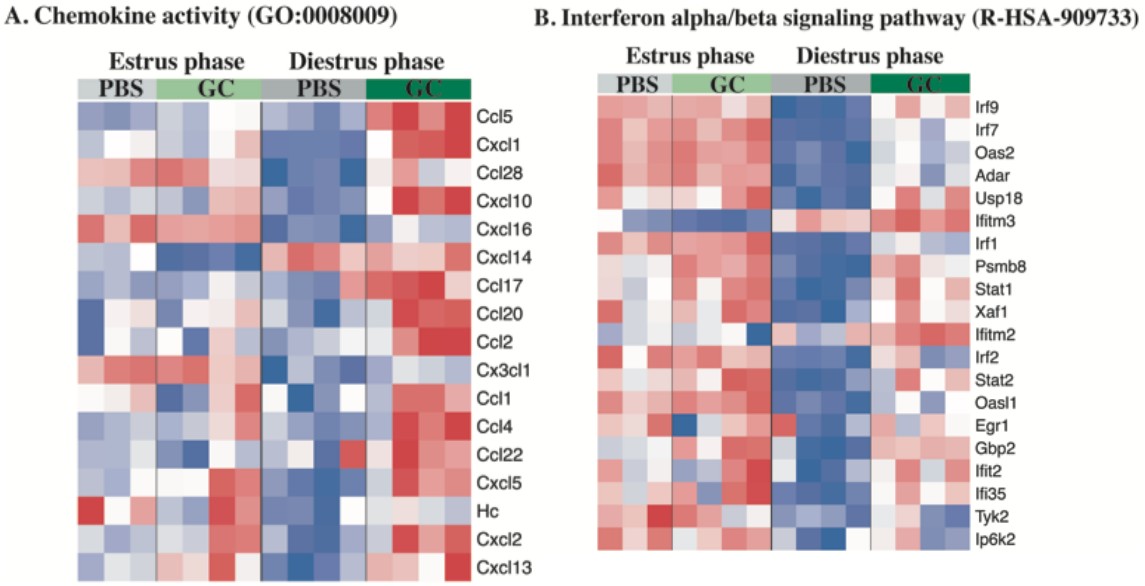


Figure 3.9: Leading edge genes from top phase:infection interaction-effect gene sets. Transcript levels of leading edge genes, from GSEA identified top phase:infection interaction effect gene sets (both FDR $q < 0.001$) (**A.** Cytokine activity gene set [GO:0008009], **B.** Interferon alpha/beta signaling pathway [R-HSA-909733]), as measured 6 hours after infection, are displayed by heatmap. Rows represent genes, with log2(expression) values z-normalized (to a mean of zero and a standard deviation of one) across all samples. Colors are scaled so that red and blue indicate z-scores of ≥ 2 or ≤ -2, respectively, and white indicates a z-score of 0 (row-wise mean). Rows are arranged in descending order from top to bottom by phase:infection *t* statistic. N=4 per condition except PBS estrus N=3

In contrast to the cytokine gene set, most of the leading-edge genes in the interferon alpha/beta signaling gene set (Reactome pathway R-HSA-909733) (Figure 3.9b), displayed very little infection dependent induction in estrus but robust diestrus-phase induction by infection. The leading-edge genes suggest a fully mature interferon response active in diestrus infected tissue with induction of positive regulators of type one interferon expression (*Irf1*, *Irf7*), signaling (*Irf9*, *Stat1* and *Stat2*), effector function (*Ifitm2*, *Ifitm3*, *Gbp2*, and *Ifi35*), and members of negative feedback control pathways (*Usp18*, *Irf2*). The activation of a type 1 interferon response by GC infection is a

relatively newly described phenomenon and its presence in our transcriptional study highlights its potential importance in the host response to gonococcal infection (Andrade et al., 2016). In the context of this study, increased activity of type 1 interferons only during diestrus infection suggests that these processes may be, in addition to the previously described differential activation of the common anti-gonococcal response, responsible for the profound phenotypic differences in GC infection at distinct reproductive cycle phases.

GC infection in diestrus phase induces greater expression fold changes of molecular components of granulocyte trafficking

We employed Ingenuity Pathway Analysis (IPA) to further evaluate the biological pathways represented in significant (FDR $q < 0.25$) phase:infection interaction effect genes. IPA identified, among other pathways, adhesion and diapedesis pathways for both granulocytes (neutrophils, basophils and eosinophils) and agranulocytes (lymphocytes and monocytes) as significantly differentially induced in diestrus infection compared to estrus infection. Since our current transcriptional analysis suggests a differential induction of a chemokine-centric common anti-gonococcal response may be partially responsible for the previously reported differences in granulocyte infiltration of infected tissue between diestrus and estrus phases, we chose to examine the granulocyte pathway more closely (Figure 3.10).

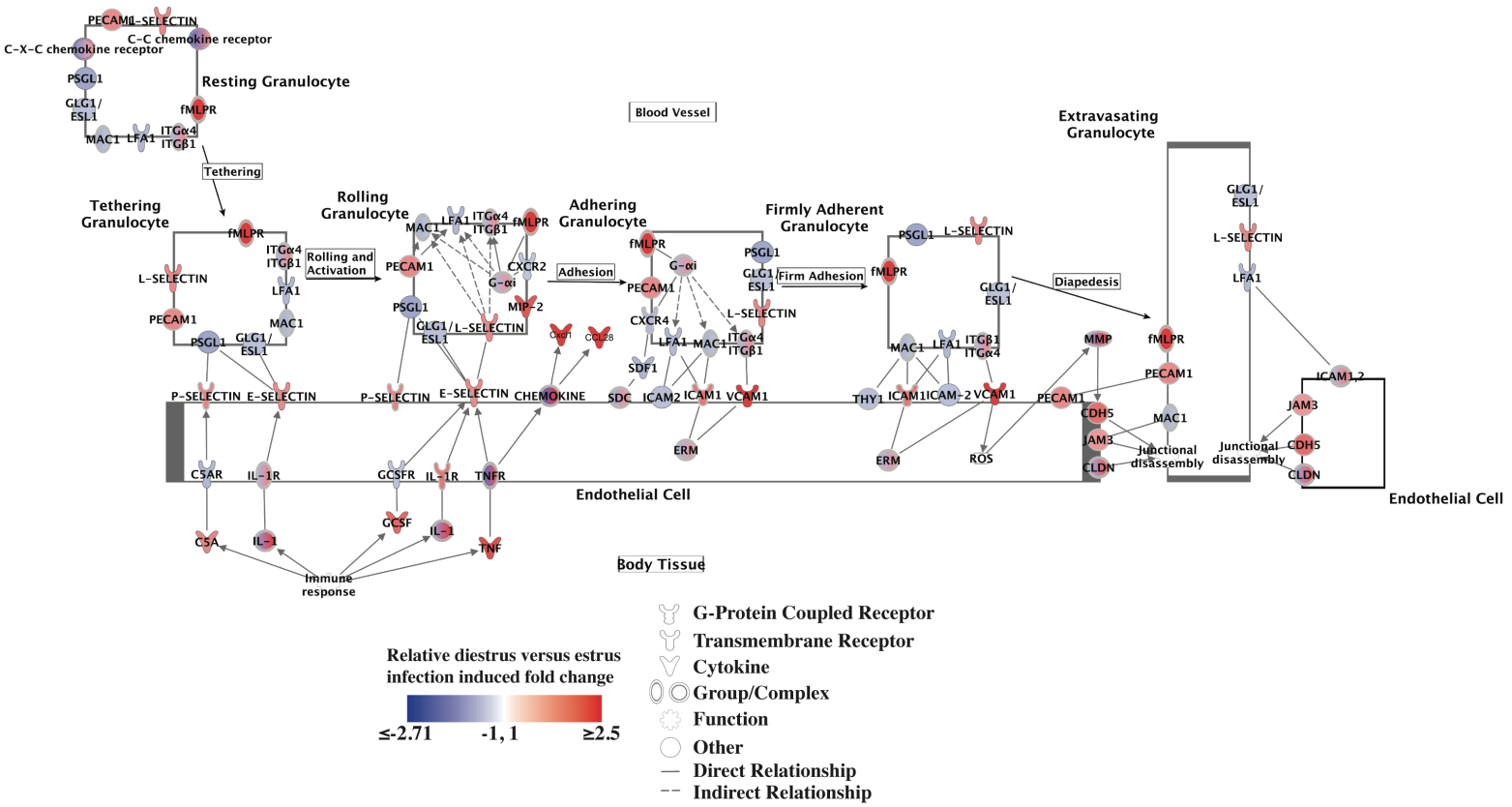


Figure 3.10: Molecular components of granulocyte activation and diapedesis are induced to a greater degree during infection in diestrus phase than in estrus phase. The members of the Ingenuity Pathway Analysis "Granulocyte Activation and Diapedesis" pathway are shaded according to the differential fold change in transcript level after 6 hours of transcervical infection with 10^7 gonococci during diestrus versus estrus phase over phase specific controls (signed ratio of infection-induced fold change in diestrus phase to that in estrus phase). Colors are scaled so that red and blue indicate differential fold changes of ≥ 2.5 or ≤ -2.71, respectively, and white indicates a differential fold change of 0 (no difference in fold change between phases). N=4 for all conditions except PBS estrus N=3

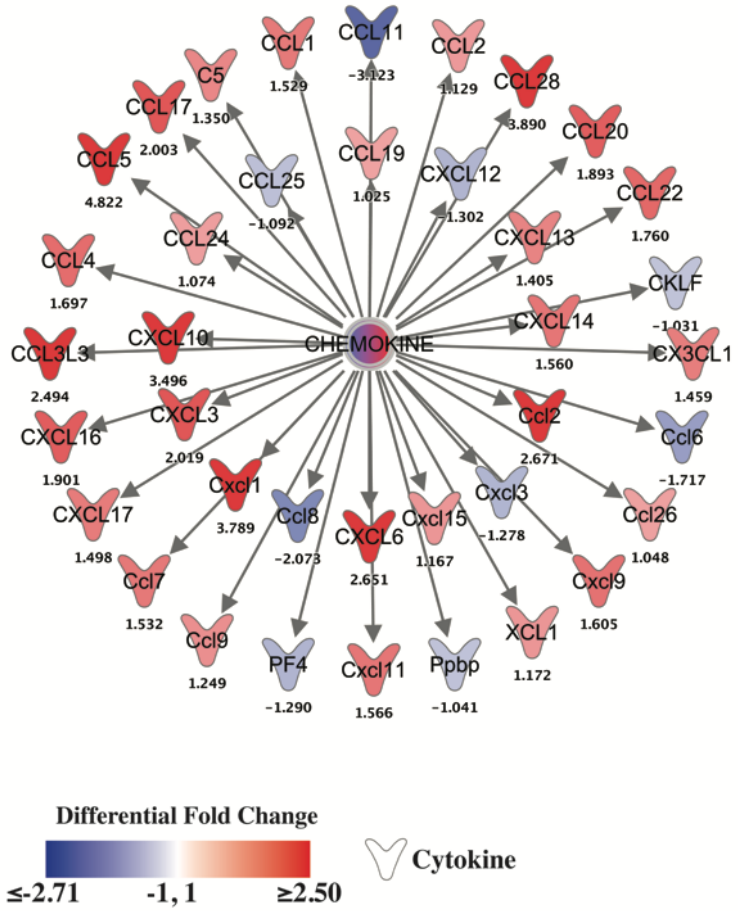


Figure 3.11: Chemokines are primarily upregulated to a greater degree during infection in diestrus phase than in estrus phase. The phase-dependent effect on infection induced chemokine expression is shown as differential fold change in transcript level after 6 hours of transcervical infection with 10^7 gonococci during diestrus versus estrus phase over phase specific controls (signed ratio of infection-induced fold change in diestrus phase to that in estrus phase). Colors are scaled so that red and blue indicate differential fold changes of ≥ 2.5 or ≤ -2.71, respectively, and white indicates a differential fold change of 0 (no difference in fold change between phases). N=4 per condition except for PBS estrus N=3

The chemokine components of the granulocyte adhesion and diapedesis pathway showed some of the greatest differences in phase-dependent infection induction (Figure 3.11), including the primary neutrophil chemokine Cxcl1 and the strong mucosal lymphocyte chemokine Ccl28 (Hieshima et al., 2003; Lazarus et al., 2003), which had differential fold change (DFC; i.e., ratio of fold change during diestrus-phase infection to fold change during estrus-phase infection) values of 3.8 and 3.9, respectively. In addition to classic chemokines, the gene Fpr2, which encodes a receptor for the potent neutrophil chemoattractant formyl-methionyl-leucyl-phenylalanine (fMLPR) (Schiffmann et al., 1975; Becker, 1987; Boulay et al., 1990; Bignold et al., 1991), is also upregulated by infection in a phase-dependent manner (DFC of 5.7). Similarly, genes involved in neutrophil rolling, adhesion (the selectins Sell and Selp and the adhesion molecules Icam1, Pecam1, and Vcam1) (Muller et al., 1993; von Andrian et al., 1993; Burns et al., 1999; Yang et al., 2005; Lomakina and Waugh, 2009; Xie et al., 2015) diapedesis and transmigration (e.g., Cdh5 and Jam3) (Chavakis et al., 2004; Wessel et al., 2014) showed greater positive induction in diestrus than estrus infection, generating DFC values ranging from 1.2-2.5. Taken together, these observations describe a highly activated endothelium interacting with a large, chemokine-mobilized population of granulocytes, leading to increased movement of cells into infected tissues. These observations help to clarify elements of the driving mechanism behind the reproductive-cycle-phase-dependent phenotypic differences in host response to GC infection.

In this study, we present evidence to suggest that the reproductive cycle has a profound effect on the transcriptomic response to uterine infection with *Neisseria*

gonorrhoeae. Although a chemokine focused induction of cytokine expression and function was observed in all infected tissue, the magnitude of this common anti-gonococcal response was phase-dependent. There was significantly greater expression of immune cell recruitment molecules, particularly those that target neutrophils, when infection occurred in the diestrus phase. In fact, the granulocyte adhesion and diapedesis biological pathway demonstrated general greater activation in diestrus phase infection as compared to estrus phase infection. This differential response likely drives the greater tissue infiltration of neutrophils during diestrus infiltration that we described in our previous work. While neutrophil infiltration was perhaps the most striking difference between infection in diestrus and estrus phase, there also appeared to be greater inflammation and loss of mucosal integrity during diestrus. Although our work here cannot fully explain the molecular cause of these additional differences we were able to identify a diestrus infection specific activation of type 1 interferon pathways. These pathways have been implicated as host-detrimental in some anti-bacterial responses which raises the question of its effect in *Neisseria gonorrhoeae* infection. These findings help clarify the underlying biological processes that characterize the anti-gonococcal response, both protective and potentially destructive, in the wild-type murine transcervical *Neisseria gonorrhoeae* infection model. However, as has already been shown, hCEACAM expression is also capable of significantly altering the natural progression of GC infection in mice.

Expression of human CEACAMs does not significantly alter the murine host response to diestrus-phase transcervical infection

Our previous studies demonstrate the significant impact of the natural reproductive cycle and expression of human CEACAMs on the characteristic and magnitude of the host response to *Neisseria gonorrhoeae* infection in mice. Since both hCEACAM expression and diestrus phase were associated with a greater inflammatory response to transcervical infection, we investigated the host response to infection in the context of both. For this experiment, 33 mice with an intact microbiome were phase locked in diestrus by DepoProvera treatment and infected or mock infected transcervically with tissue collected from mice at the indicated time points; 0hpi (baseline measurements on untreated mice at the time of infection), 6hpi, or 24hpi.

Surprisingly, the observed cytokine profiles were remarkably similar in both magnitude and character between all three genotypes (Figure 3.12). Minor differences in magnitude are seen with a few cytokines however the overriding similarity is the most notable element of these results. This suggest that the potent inflammation that is observed in diestrus phase transcervical infection is induced by processes independent from bacterial adhesion, in the case of hCEACAM1 mice, or neutrophil interaction in the case of CEABAC2 mice. Our collaborators have described increased uterine tissue infiltration in diestrus transcervical infection that occurs in a hCEACAM independent manner. It may be that this mass tissue invasion is adequate to induce the strong inflammation of the diestrus transcervical infection. These results underscore that it is not

just the reproductive phase or the presence of gonococcal adhesion targets that dictate the pathological potential of an infection but a complex interplay between the two.

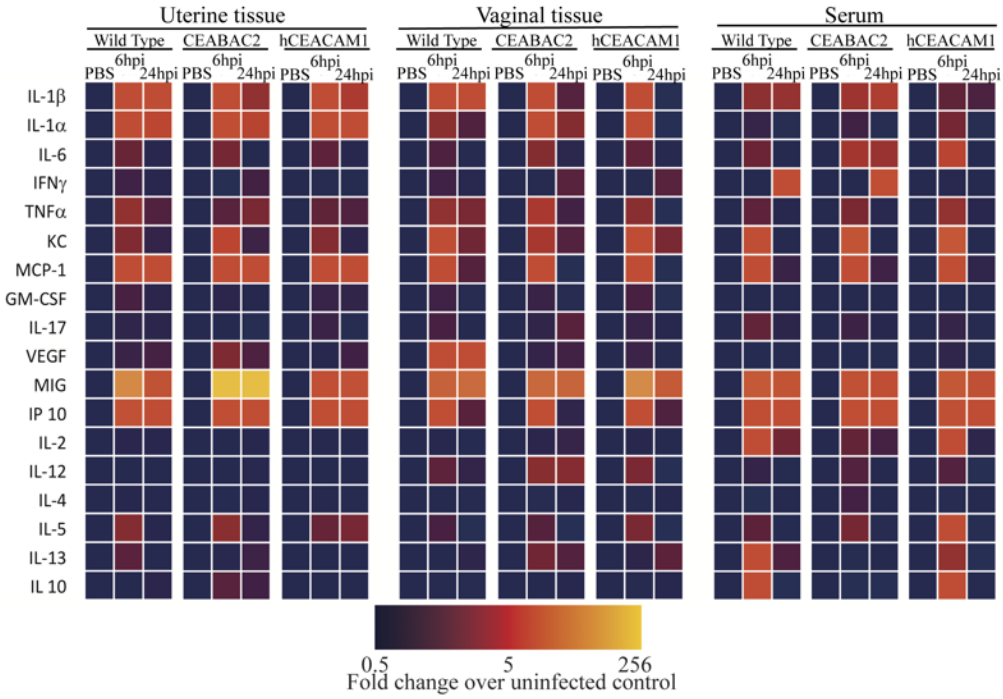


Figure 3.12: Host response to transcervical GC infection in diestrus phase, as measured by cytokine induction, is not impacted by hCEACAM expression. "Mice treated with DepoProvera were infected transcervically with OpaCEA-expressing Ngo. Levels of cytokines in upper, lower genital tract homogenates, and sera samples were measured by LUMINEX multiplex assays at the indicated time points. Heat maps were generated to depict fold change normalized to uninfected PBS controls for 6 h. n=3 of each genotype per time point for infected groups, n=2-3 per genotype for uninfected PBS controls. Heat map was generated in Microsoft Excel." Recreated with permission here, in part, from (Islam et al., 2016)

Discussion

For more than two decades, the primary laboratory model of *Neisseria gonorrhoeae* has been the intravaginal infected estrus mouse, which is permissive to colonization only during the estrus phase of the reproductive cycle. In this model, mice develop a mild inflammation of the vaginal cavity and allow bacterial persistence only as

long as estrus phase is maintained through exogenous estradiol and the microbiome is suppressed by antibiotic treatment (Packiam et al., 2010). This model recreates much of the phenotype associated with asymptomatic human infection, including mild inflammation, along with a failure to induce an adaptive immune response and immunologic memory (Packiam et al., 2010; Jerse et al., 2011). Where this model falls short, is in the production of active infection. It is well characterized that *Neisseria gonorrhoeae* does cause symptomatic lower FGT disease as well as ascends to the upper FGT in a subset of all infected individuals, symptomatic or not (Barlow and Phillips, 1978; Soper et al., 1994). The estrus model has not shown an ability to model either of these conditions in mice. A model able to recapitulate the biology and phenotype of the symptomatic phases of human gonorrhea is of increased importance with the push for new pharmaceuticals to treat or prevent these active pathologies. Based on human and non-human studies we identified the expression of bacterial adhesion targets and the natural reproductive cycle as variables that might impact the natural progression of GC infection in mice, and model symptomatic human gonococcal infections.

The results presented here suggest a complex relationship between adhesion target expression, reproductive cycle, and site of primary infection. Our collaborators reported an increase in length of vaginal gonococcal carriage following estrus vaginal infection in FGT hCEACAM expressing mice (CEABAC2 mice) but this did not correspond with increased inflammation (Figure 3.3). In contrast, any hCEACAM expression resulted in a more active infection and inflammatory response in the context of transcervical estrus infection (Figure 3.4). Surprisingly, it was the expression of neutrophil receptor

hCEACAM3 rather than the presence of the upper FGT adhesion target, hCEACAM1 that resulted in the greatest inflammation and immune activation. As opposed to the estrus model and the wild-type transcervically infected mice, hCEACAM expressing mice demonstrated proximal tissue inflammation in isolated vaginal tissue and the hCEACAM 3, 5, and 6 expressing CEABAC2 mice had a robust systemic chemokine response (Figure 3.5). These two studies show that the contribution of hCEACAMs to the development of inflammation in murine infection is not just as adhesion targets. The immunologically active hCEACAM3 appears to be a powerful inducer of inflammation within and beyond the site of primary infection. This response overwhelms the effect of adhesion expression, which is significant in its own right. Perhaps most importantly these studies show that upper FGT gonococcal infection in mice is possible, however is dependent upon the direct instillation of bacteria into the uterus.

As mentioned, PID appears to have a significant connection to the human menstrual cycle. A similar effect was described here in mice and was found to be hCEACAM independent (Figure 3.12). Direct transcervical infection of the upper genital tract during diestrus induced a powerful proinflammatory cytokine and chemokine response (Figure 3.6). Upon examination of the tissue, our collaborators described an overt uterine pathology, including inflammation, granulocyte invasion, disruption of the epithelia and clinical signs of distress (Islam et al., 2016). Interestingly, the clinical and histological pathology was absent or significantly reduced in infection during estrus.

A detailed analysis of the host transcriptional response to transcervical infection in estrus and diestrus phase showed identified induction of primarily chemokine

pathways as by GC infection. Due to the nature of our analysis, we were able to evaluate differential expression and therefore biological pathway activation due to the independent effect of just infection status, or due to a combined phase:infection interaction effect. It was through this analysis that we were able to show that a very similar profile of biological pathways is induced in response to GC infection regardless of during which reproductive phase infection occurs. This observation is significant in light of our previously described profound differences in infection phenotype during diestrus phase and estrus phase. This suggests that, despite the different natural histories of infection that have been described, a chemokine-centric induction of cytokines may be the foundation of a common anti-gonococcal host response. Reflecting this, leading-edge genes from the "Chemokine Receptors Bind Chemokines" pathway, identified by infection effect GSEA, demonstrate clear phase independent induction (Figure 3.7).

Interestingly, this common anti-gonococcal program has greater activity in diestrus infection. Several of the same chemokines identified by infection effect GSEA, are found among the leading-edge genes of a top phase:infection interaction effect GSEA gene set, "Chemokine activity" (Figure 3.9a). Additionally, closer examination of the leading-edge genes of significant chemokine gene sets shows significant representation of neutrophil chemokines (Cxcl5, Ccl4 and Cxcl1) suggesting strong phase:infection interaction effect. Supporting this, the phase:infection effect clustering showed that several potent neutrophil chemokines demonstrated a diestrus specific induction expression pattern (Figure 3.8c). This would explain, at least in part, the significant differences in neutrophil recruitment during GC infection in different reproductive cycle

phases described by our collaborators (Islam et al., 2016). The model of granulocyte adhesion and diapedesis was able to confirm, not only greater activation of the pathway during diestrus infection, but that chemokines and endothelial mediators of diapedesis most contribute to this difference (Figures 3.10 & 3.11). This general anti-GC response, in addition to reflecting the human and mouse data found in the literature, is reminiscent of the clinical picture of PID, characterized by intense local inflammation and influx of granulocytes into infected tissue.

In contrast to the common induction of chemokine activity during GC infection, the induction of interferon pathways appears to be largely unique to GC infection in diestrus phase. Although the activation of these pathways during GC infection has been reported before (Dobson-Belaire et al., 2010; Andrade et al., 2016), it is a phenomenon that is much less understood than the previously discussed cytokine response. Those studies that have examined the impact of type 1 interferons in GC infection have suggested, based on impaired bacterial killing in the context of IFN-β, that it has a detrimental effect on infection control and resolution (Andrade et al., 2016). A negative impact of type 1 interferon on an antibacterial response has been described for several other human pathogens including the genito-urinary pathogen *Chlamydia trachomatis* (Qiu et al., 2008). While the role of type 1 interferon signaling in GC infection is not yet fully understood, the significant and specific induction of related pathways in diestrus phase infection, where greater pathology is observed, suggests a possible detrimental effect on the host. The emergence of these immune processes in our transcriptional

analysis reinforces their potential importance and supports the further exploration of the role of type 1 interferons in human gonorrhea and the murine model of disease.

Together, this body of work shows that the previous theory of murine susceptibility to GC infection was overly simplistic. Infection and the subsequent host response is strongly dependent on the time and site of infection. This is demonstrated by observations that the wild type vaginal tract is unresponsive to infection in diestrus and reactive in estrus while the uterus demonstrates the complete opposite pattern. Additionally, we show that the expression of hCEACAMs 1, 3, 5, and 6 play a significant role in the progression of estrus phase infection, both vaginal and uterine. Based on these results we suggest the further characterization of the transcervical diestrus infected mouse as a laboratory model of PID, as well as its use in parallel with the vaginally infected estrus CEABAC2 mouse as a new model of subclinical gonorrhea.

Limitations of the studies

In contrast to all other presented studies, the mice in the study that compared transcervical infection in estrus and diestrus were not arrested in their given reproductive phase by exogenous hormones. The intermittent use of these treatments may have unforeseen impacts on the observed responses to infection. The induction of estrus and diestrus phases by β-estradiol and DepoProvera respectively are well-established protocols thought to closely mimic the natural reproductive phases. Despite this, both of these hormones can influence immune function and their exogenous addition cannot be ruled out as a possible confounding variable without further study. A small experiment

comparing the infection induced cytokine/chemokine response in naturally cycling wild type mice with those in DepoProvera and β-estradiol induced reproductive phases would clarify any impact this variable had on our observations.

As mentioned earlier, no antibiotic treatment is needed during transcervical infections because there is no risk of out-competition of GC by commensal microbes. For all vaginal infections however, mice are treated for an extended period with collection of broadly reactive antibiotics. While data is not available, this treatment must have wide sweeping effects not just on the microbiome of the FGT but of the gut and other body sites as well. These changes very well could have strong influences on the reactogenicity of the immune system. The field of microbiome immunology is expanding quickly and has repeatedly shown that host immune function is profoundly influenced by the composition and status of the microbiome (Koeberling et al., 2009; Belkaid and Hand, 2014; Sherwani et al., 2018). As the models currently stand, this antibiotic treatment is still necessary for murine vaginal infection, however it must be recognized as a potentially serious confounding effect.

Finally, these studies, along with those of our collaborators rely upon a largely phenotypic description of human gonococcal infection, particularly with regards to gonococcal PID, to guide the development of our disease models. Studies examining human gonorrhea have largely been limited to serum cytokines and the more comprehensive studies have looked only in men (McCormack et al., 1977b; Ramsey et al., 1994). The ideal *in vivo* lab model is one that recreates the biology of the system is meant to mimic. In this case, we don't have a solid handle of the biology of human

gonorrhea and therefore must rely upon the recreation of its phenotype. The resolution of this limitation is exceedingly difficult because to better understand human gonorrhea would require the collection and study of tissue from actively infected individuals. To achieve this, while also fulfilling our responsibilities to the patient, would be difficult. A potential solution is to examine the gonococci in the context of infection rather than the host. Where a tissue sample is invasive and requires significant logistical support to collect, extracting microbial RNA from a simple swab of an infected site would provide a snapshot of the pathogen in its infectious form. This could be done in both humans and mice and by comparing the two microbial transcriptional profiles we could potentially determine if from the gonococcus' perspective, our models of gonorrhea appear similar to the real thing.

Chapter 4: Naturally released outer membrane vesicles derived from an RMP-deficient strain of GC, demonstrate consistent composition and are strong, poly-immunogenic anti-gonococcal vaccine antigens

Introduction

Outer membrane vesicles derived from Gram-negative bacteria are not new to the field of microbiology (Chatterjee and Das, 1967), however, they have only recently become objects of intense interest in the fields of biomedical engineering and vaccinology (Zhu et al., 2005; van de Waterbeemd et al., 2013; Acevedo et al., 2014; van der Pol et al., 2015; Lee et al., 2016). It is easy to see the allure of OMVs from the perspective of vaccine antigen development. The vesicles, by definition, contain a large contingent of outer membrane structures in their native conformation (Kulp and Kuehn, 2010). These structures are the typical antigenic targets of vaccine design since critical metabolite/waste exchange processes and bacterial adhesion mechanisms are mediated by extracellularly exposed structures embedded in the outer membrane. The targeting of these antigens by the immune system results in microbial inhibition and death by both disrupting necessary metabolic functions and through direct immune mediated bactericidal action (Jerse and Deal, 2013; Wetzler, 2014). In addition to their antigenic cargo, OMVs contain an array of PAMPs that could allow the vesicles to function as self-adjuvanting antigens. This characteristic could possible eschew the need for an exogenous adjuvant, many of which are known to induce very unidimensional immune responses. For example, the adjuvant MPL-a induces a very strong Th1/cell-mediated-immunity biased response, whereas alum salts produce a strongly Th2 biased humoral

immunity (Korsholm et al., 2010). Of course, when utilizing naturally produced OMVs (nOMVs) there is always the concern that the vesicles may have an immune-inhibiting endogenous function that, as a vaccine antigen would result in, at best, poor immunological memory and at worst greater susceptibility to infection and disease.

Already, a GC-protective mechanism induced by an antigen has been identified in the anti-RMP response. As previously described, early trials of a GC porin vaccine were deemed to be non-protective (Tramont, 1989) and some evidence suggested that immunized individuals were more likely to subsequently develop disease (Plummer et al., 1993). When samples from study participants were analyzed, it was determined that, while RMP was a minor antigen in the vaccine, it was the dominant immunogen. Unfortunately, the anti-RMP antibodies had no bactericidal activity and they also disrupted the bactericidal activity of the anti-porin antibodies (Gulati et al., 1991; Rice et al., 1994). The mechanism of this phenomenon is not entirely clear; however, it has been shown that the decrease in bactericidal activity in the context of anti-rmp antibodies, occurs despite increased deposition of C3b and C9 on the bacterial surface (Joiner et al., 1985). These observations have led to the hypothesis that the anti-rmp antibodies somehow divert the activated complement components to “non-bactericidal sites” on the bacteria. For this reason, all GC vaccines must exclude RMP completely. This is particularly difficult with an OMV vaccine since RMP is a natural component of the outer membrane. As the selective extraction of RMP from the bacterial or vesicular membrane would prove impossible, we must isolate nOMVs from GC that don’t express RMP. Our group had previously constructed and described such a strain while optimizing

the purification of P1B (Wetzler et al., 1989). It is that strain, constructed in the MS11 strain of GC, that will be used to explore the potential of RMP deficient nOMVs.

Beyond a possible RMP-mediated bacterioprotective mechanism, little is known about the functions or effects of *Neisseria gonorrhoeae* nOMVs *in vivo*. In fact, GC nOMVs have yet to be identified in infection. There have been a few *in vitro* characterizations of wild-type GC nOMVs which show that they contain a diverse array of outer membrane proteins, several of which are likely critical for human virulence (Zielke et al., 2014). However, the best insights we may have into the potential *in vivo* effects of GC nOMVs is through studies on vesicles produced by *Neisseria meningitidis* (Nm). The capsular polysaccharide of group B Nm has a high degree of antigenic similarity to endogenous human molecules (Finne et al., 1983) necessitating a non-capsule-conjugate vaccine. Researchers discovered that group B derived Nm OMVs successfully induced protective immunity against the strain and bypassed the potential risks of the capsular vaccine (Granoff, 2009). Through the studies of these Nm OMV vaccines we know that the vesicles are immunostimulatory, composed of a consistent array of critical outer membrane associated structures, and are highly stable (Granoff, 2010). Most importantly, Nm OMVs are highly immunogenic and produce protective immunity against meningococcal disease. To fully evaluate the vaccine potential of GC nOMVs, we must examine the strength and characteristics of the *in vivo* anti-nOMV immune response.

Protective adaptive immunity in the genitourinary tract, male or female, is mechanistically poorly understood. With mucosal surfaces, like with all externally facing

tissue, physical protection afforded by the epithelia is important. This is clear from the studies we presented in Chapter 3, where the simple, non-glandular uterine-lining of diestrus phase corresponded with increased gonococcal tissue penetration and subsequent inflammation. Beyond physical barriers, there is a significant role for secreted immunoglobulin in the protection of mucosal sites, particularly by luminal neutralization of pathogens. This has been appreciated since 1919 when Alexandre Besredka showed that protection of rabbits against dysentery following oral *Shigella* immunization was unrelated to serum antibody titers (Besredka, 1919). This phenomenon was then described in humans where anti-*Shigella* antibodies were measurable in stool before they were measurable in sera (Davies, 1922). Proof that these secreted immunoglobulins executed their effector functions in the lumen came from studies of newborn piglets showing that the dissemination of enteric commensals and subsequent septicemia was prevented by luminally restricted Ig (Rejnek et al., 1968).

As the field has advanced we have come to recognize that the secreted mediators of this immunity are IgA, IgM and IgG (Brandtzaeg, 2013). The classic mucosal immunoglobulin (Ig), secreted IgA (sIgA), has been thought to be the primary effector immunoglobulin of mucosal immunity (Brandtzaeg, 2013). This designation, though, came during the early development of the field of mucosal immunology when the vast majority of studies focused on the gut. As mucosal immunologists work to develop our understanding other mucosal sites, there is growing evidence that the primary effector immunoglobulins in mucosal sites may depend on where and when you look. This is particularly true in the female genital tract (FGT) where protective mechanisms for some

pathogens have been shown to be IgG not IgA mediated (Parr and Parr, 1997), and where the relative levels of the different Ig classes are highly dynamic and linked to the reproductive cycle (Usala et al., 1989; Nardelli-Haefliger et al., 1999). Several publications even suggest that IgG is the dominant and most important Ig to FGT protection (Parr and Parr, 1997; Johansson and Lycke, 2003; Li et al., 2011).

While our grasp of the mechanistic details of mucosal immunology lags behind that of the systemic immune system, our understanding of how to induce protective immune memory at specific mucosal sites is even worse. This could be because, while the idea that systemic and mucosal immunity are distinct from one another is not new, we don't yet fully appreciate the implications of this distinction. For instance, despite the majority of vaccine preventable diseases being mucosal diseases or dependent upon mucosal transmission, most vaccines are given parenterally (IAC, 2018). It is well established that the route of exposure to an antigen heavily biases the site of induced memory; particularly with regards to mucosal exposure and mucosal immunity (Parr et al., 1988; Brandtzaeg, 2007). This would suggest that these systemic vaccines are capable of overcoming this barrier to mucosal protection following systemic exposure, the protection is dependent on systemic and not mucosal immunity, or that protection at mucosal sites is a case of luck rather than effective design. The reality is likely a mixture of all three, with the potent adjuvants used today, we know that the intense adaptive response that is induced by vaccination can spill over into the mucosal immune system like in the case of the HPV vaccine (Mariani and Venuti, 2010).

Unfortunately, many of the mucosal diseases for which we most need vaccines, have proven to be resistant to current standards of vaccine development including the modern array of adjuvants (Leroux-Roels, 2010). While new, mucosally-directed, adjuvants are being developed (Hasegawa et al., 2009; Fukuyama et al., 2015), it is important to also consider simple interventions that might increase the mucosal immunogenicity a vaccine, like route of delivery. Within the mucosal immune system there appear to be specific tissues that are more closely linked than others, which can impact the site and magnitude of immune response to a vaccine (Parr et al., 1988; Brandtzaeg, 2007). These studies have described a strong link within the common mucosal immune system between the naso-oropharyngeal and FGT mucosa (Belyakov and Ahlers, 2009). This link may open the door to effecting induction of tissue specific immunity in the FGT which is a major challenge to the successful development of an anti-gonococcal vaccine for women. Unsurprisingly since the anatomy is radically different, immunological protection of the male genital tract appears to be different from FGT (Russell and Mestecky, 2002). Primary protection appears to be from mechanical barriers (urine flow and epithelial shedding) as well as serum derived IgG and locally produced sIgA (Mestecky and Fultz, 1999). For the development of an anti-gonococcal vaccine, we focus on the induction of FGT immunity since as of now no male model of infection exists in which to test a potential male GC vaccine.

Achieving strong, mucosal based anti-gonococcal immunity is a significant challenge. The mechanisms of protective against the pathogen are not known and the methods for inducing immunity in the mucosal tissues most critical for this STI are not

understood. However, as discussed above, there is evidence to suggest that a poly-antigenic vaccine, like an OMV could overcome some of the obstacles and induce anti-gonococcal protection. We believe that nOMVs derived from a Δ*rmp* mutant GC strain would best address the challenges of an anti-gonococcal vaccine. In the following studies, we demonstrate the isolation and *in vitro* characterization of Δ*rmp* GC nOMVs followed by the *in vivo* exploration of their immunogenicity and how it is influenced by vaccine dose and route of delivery.

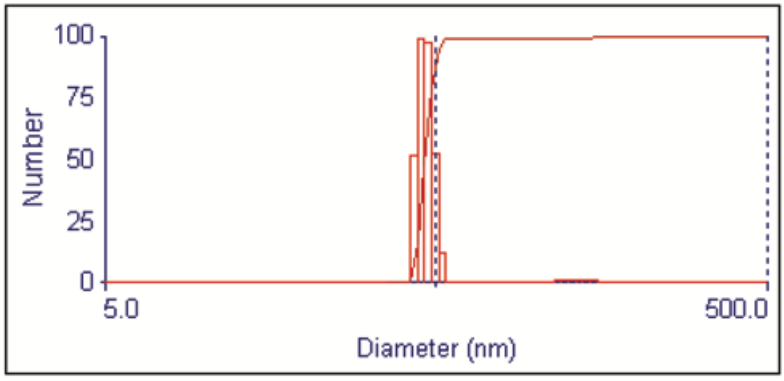
Results

Naturally released outer membrane vesicles can be isolated from an *rmp* deficient strain of *Neisseria gonorrhoeae*

Many researchers have previously isolated and utilized gonococcal OMVs (Evans, 1977), however, it is possible that the deletion of the RMP protein could affect the natural production of vesicles. This protein shares significant homology to the *E. coli* OmpA protein (UniProtKB ID#F8S842), which among other things, helps to anchor the outer membrane to the peptidylglycan wall (Wang, 2002). In fact, genetic deletion of the OmpA coding gene *ompA* is known to alter the production rate and composition of *E. coli* nOMVs (Sonntag et al., 1978). For this reason, we first examined whether nOMVs could be isolated from an RMP minus GC strain by a similar process as from wild-type strains, and whether they are structurally and physiochemically similar.

In order to isolate RMP-deficient GC nOMVs we utilized sequential size restrictive filtration, to remove large debris and cells, and subsequent weight restrictive

stirred ultrafiltration to retain large molecular weight structures (nOMVs) from a large volume liquid culture of a *rmp*-deletion mutant strain of *Neisseria gonorrhoeae* (GCΔ*rmp*) (see methods for details). Initial characterization of the resulting material was done by Dynamic Light Scattering (DLS), a method that utilizes the theory of Brownian motion to predict particle size based on the scattering of incident light. Per DLS (Figure 4.1) the isolated particles were largely one single population roughly normally distributed around a diameter of 47nm. Since DLS assumes particles are spherical and are suspended in an ideal liquid, definitive size and integrity must be investigated by a secondary method.



d(nm)	G(d)	C(d)	d(nm)	G(d)	C(d)	d(nm)	G(d)	C(d)
36.8	0	0	63.7	0	99	110.2	0	99
38.7	0	0	66.9	0	99	115.8	0	99
40.6	0	0	70.3	0	99	121.7	1	100
42.7	51	16	73.9	0	99	128.0	1	100
44.9	100	48	77.7	0	99	134.5	1	100
47.2	98	79	81.7	0	99	141.4	0	100
49.6	52	96	85.9	0	99	148.6	0	100
52.2	12	99	90.3	0	99	156.2	0	100
54.8	0	99	94.9	0	99	164.2	0	100
57.6	0	99	99.7	0	99	172.6	0	100
60.6	0	99	104.8	0	99	181.4	0	100

Figure 4.1: nOMVs isolated from Δ*rmp Neisseria gonorrhoeae* appear to be a single population with a narrow range of diameters by dynamic light scattering. Approximately 1 mL of resulting material from a single isolation protocol, suspended in a sterile 3% sucrose, 0.2M glycine solution, was analyzed by dynamic light scattering (DLS). Based on light scattering, DLS predicted a single population of particles with a narrow normal distribution of diameters centered around ~45nm. d(nm) is diameters in nanometers, G(d) is relative percentage contribution of the size range, C(d) is cumulated percentage contribution.

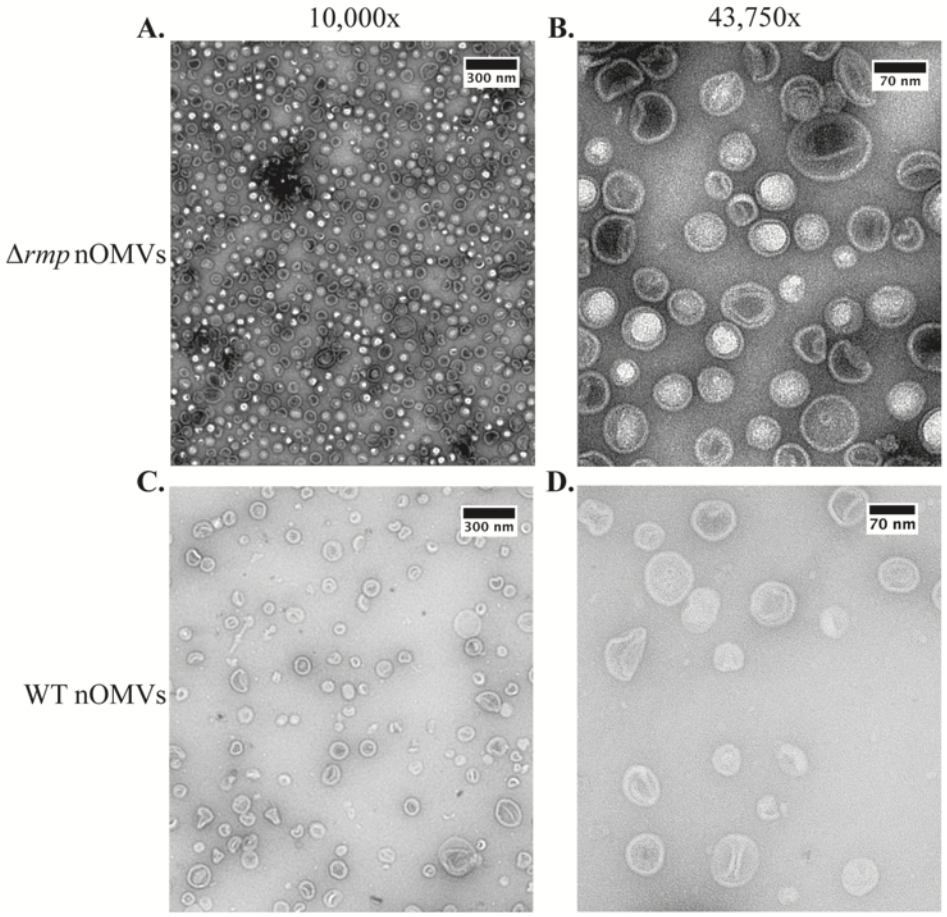


Figure 4.2: nOMVs derived from Δ*rmp* *Neisseria gonorrhoeae* have similar morphology to those derived from wild type GC. 10,000x (**A.** and **C.**) and 42,750x (**B.** and **D.**) images of concentrated large molecular weight structures isolated from Δ*rmp* (**A.** and **B.**) or wild-type (**C.** and **D.**) *N. gonorrhoeae* in late log phase growth. Samples were negatively stained with ~2% Uranyl-acetate and visualized via TEM. Images were captured with CCD camera (**A.**) or build in plate camera (**B.**). Color differences are due to differences in image capture. Images were processed and scale bars generated in ImageJ.

To confirm isolation of vesicles, samples were visualized by uranyl-acetate (UA) negative stain transmission electron microscopy (TEM) (see methods for details). Wild type nOMVs were also isolated and used a reference for the Δ*rmp* nOMVs (Figure 4.2). The electron micrographs of both Δ*rmp* and wild-type nOMVs show a largely homogenous population of spherical structures that range in diameter from ~50-100nm.

No obvious morphological differences can be seen between the two genotypes. All vesicles are single lipid-bilayer bound as shown by an average membrane thickness around 6nm (ImageJ, NIH) (Kutchel and Ralston, 1988). While there was a difference in average membrane thickness between Δ*rmp* (5.95nm) and wild type vesicles (6.67nm) this difference was non-significant as determined by unpaired *t* test (Figure 4.3). Interestingly, the vesicle diameters of wild-type and Δ*rmp* nOMVs were roughly normally distributed with means close to one another (87.5nm for wild type and 77. 8nm for Δ*rmp*) but statistically different (Figure 4.4).

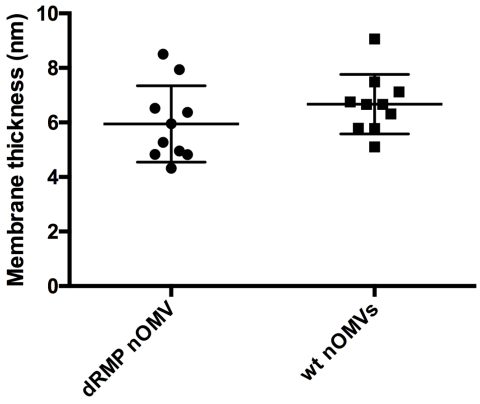
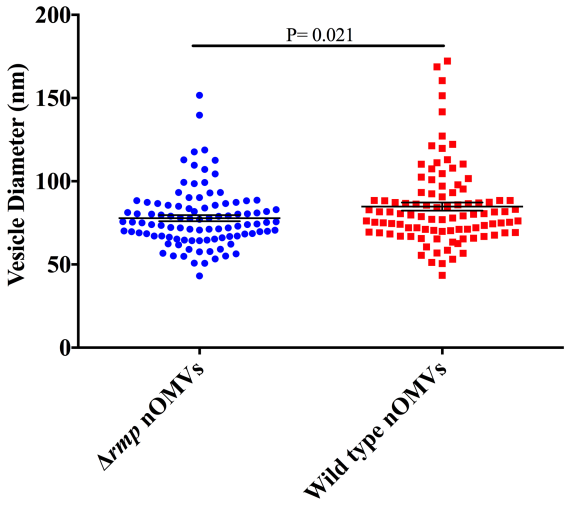


Figure 4.3: Vesicles derived from Δ*rmp* and wild-type *Neisseria gonorrhoeae* have similar thickness lipid bilayers. Membrane thicknesses of ten representative vesicles from each population were measured using ImageJ. Average thicknesses were compared by unpaired parametric *t* test using Prism by Graphpad. Whiskers indicate mean and standard deviation. Vesicles from one Δ*rmp* isolation and one wild-type isolation were compared.

Figure 4.4: Vesicles isolated from wild-type *Neisseria gonorrhoeae* are significantly larger than those isolated from Δ*rmp* GC. The diameters of 100 vesicles released by either wild-type GC or Δ*rmp* GC during concurrent but separate isolations were measured from 8,000x negative stained UA electron micrographs using ImageJ. Figure was generated and data was analyzed by unpaired *t* test in Prism. Figure whiskers indicate mean and standard error.



These results confirm that nOMVs can indeed be isolated from a *Neisseria gonorrhoeae* strain lacking the outer membrane anchor protein RMP. Isolated vesicles demonstrated a standard spherical morphology with a standard lipid bilayer. Despite a small but significant difference in vesicle diameter, there were strong similarities in other characteristics between the Δ*rmp* and wild-type vesicles further supporting the positive identification of these structures as nOMVs. Since these findings were all generated from a single isolation of Δ*rmp* nOMVs, and as discussed, consistency in these potential antigens is critical, a study of inter-batch variability is necessary.

Gonococcal *rmp* deficient nOMVs demonstrate limited inter-batch variability

To determine if there was any inter-batch variability in Δ*rmp* nOMV morphology samples from 8 separate growths and isolations were visualized by negative UA stain TEM (Figure 4.5a-h). These electron micrographs were taken an average of 4 months post isolation (range 0.5-7 months) and were mounted at varying concentrations ranging from 1 to 4 μg nOMV associated protein per μl of preparation. As shown by Figure 4.5a-h, Δ*rmp* nOMVs have a standard spherical morphology that remains unchanged between isolations. Figure 4.5i, confirms that isolated vesicles are of a standard diameter. This consistency suggests that these structures are the actively produced nOMVs that we hoped to isolate. We would expect that, if we were instead isolating products of cellular degradation, the morphology and size would be more varied.

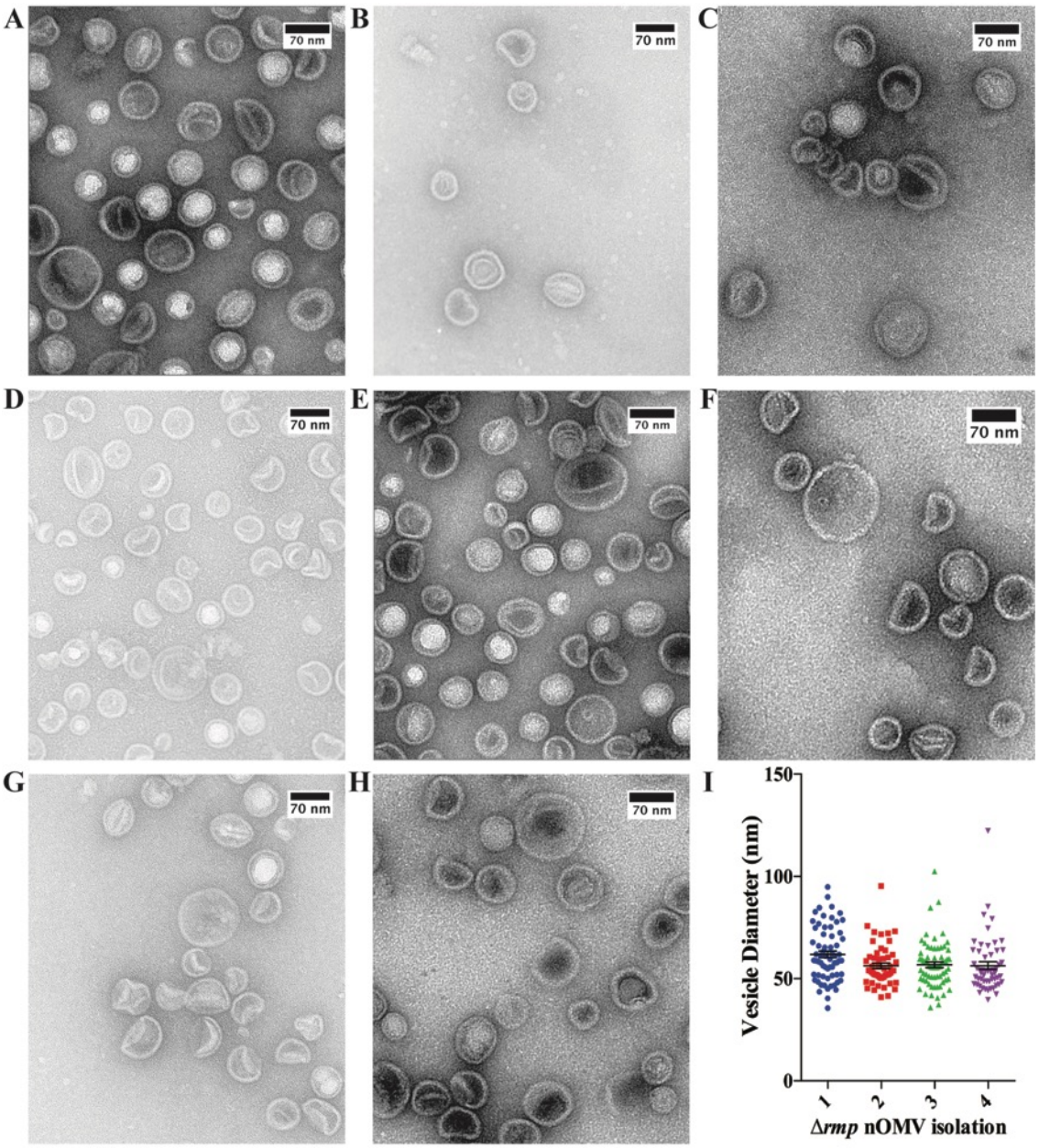
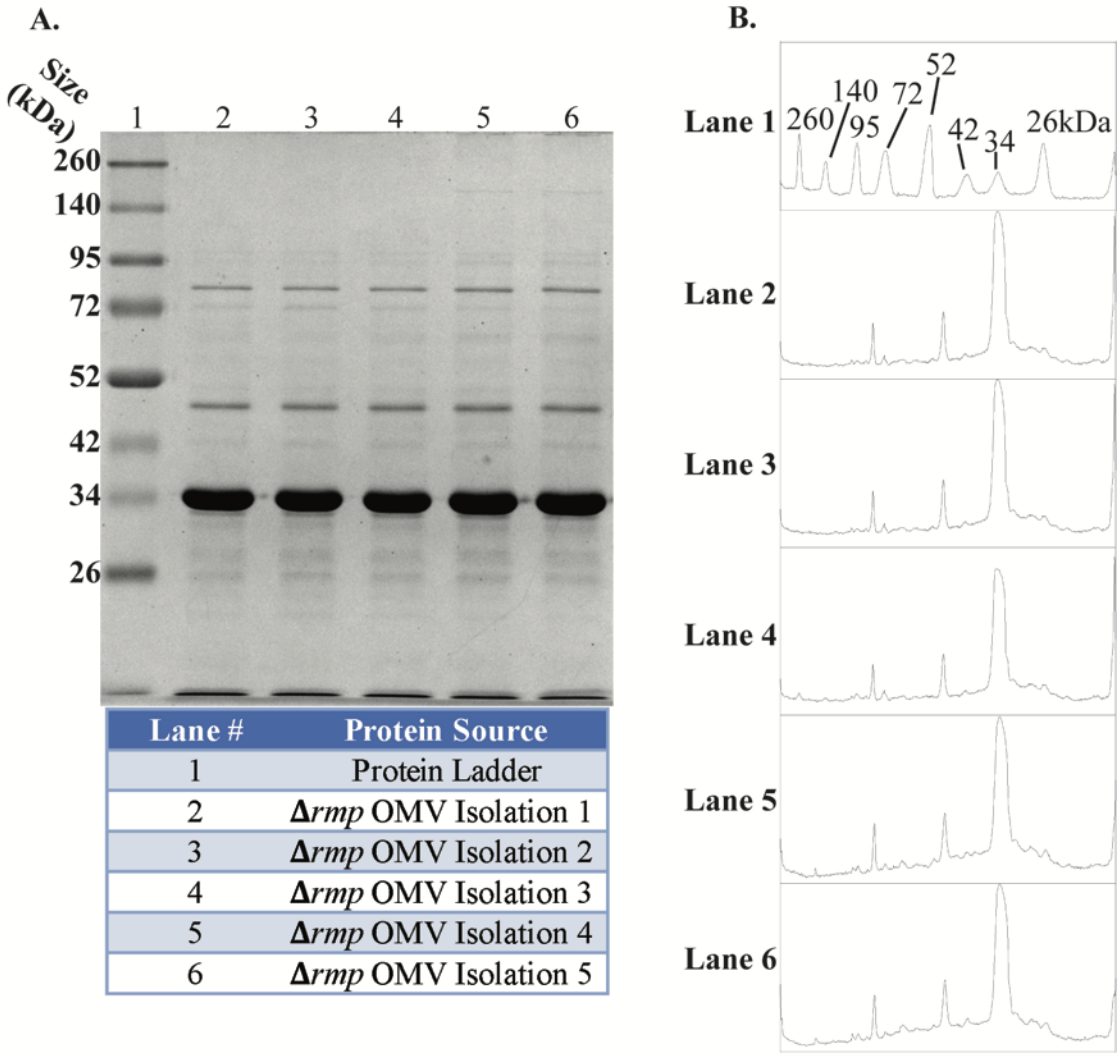


Figure 4.5: nOMVs isolated from Δ*rmp* GC have a consistent structure, morphology and size between separate isolations. Vesicles were isolated from large volume growths of Δ*rmp Neisseria gonorrhoeae* in late log growth phase. Electron micrographs (**A-I**) are representative images of vesicles each from a different isolation and growth. All samples were negative stained with ~2% UA and 43,750x EM images were captured by CCD camera (**A, C, E, F, H**) or built in plate camera (**B, D, G**). Images were processed and scale bars generated in ImageJ. **I.** The diameter of 53-67 representative vesicles from 4 separate isolations were measured by ImageJ; data was analyzed and graphed in Prism. Plot whiskers indicate mean and standard error.

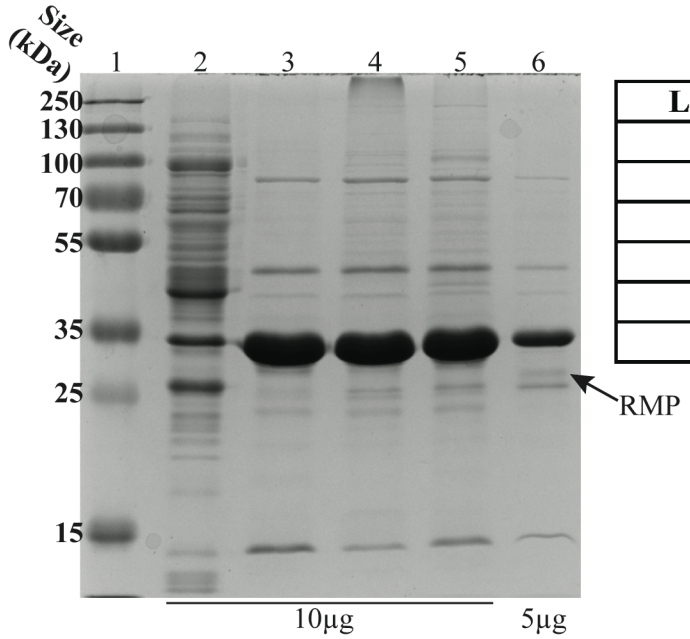


Lane #	Protein Source
1	Protein Ladder
2	Δ*rmp* OMV Isolation 1
3	Δ*rmp* OMV Isolation 2
4	Δ*rmp* OMV Isolation 3
5	Δ*rmp* OMV Isolation 4
6	Δ*rmp* OMV Isolation 5

Figure 4.6: Protein composition of Δ*rmp* nOMVs appears to be consistent between isolations. Proteins (5μg as measured by modified Lowry protein assay) from Δ*rmp* nOMVs were separated by denaturing polyacrylamide gel-electrophoresis (PAGE). Lane 1 contains the Spectra™ broad range protein ladder from ThermoFisher (Cat#26634). Lanes 2-6 each contain protein from a different nOMV isolation. **A.** Coomassie blue stained polyacrylamide gel. **B.** The protein banding pattern of each lane from the stained gel was evaluated by ImageJ and visualized by a histogram of pixel density. Moving left to right along the histogram corresponds with high to low molecular weight protein bands.

After successfully isolating Δ*rmp* nOMVs, characterizing their structure, and confirming their similarity to wild-type vesicles, we then examined their molecular composition. nOMV associated protein was extracted from 5 different isolations of Δ*rmp* nOMVs and 5μg from each was separated by molecular weight by denaturing gel electrophoresis (Figure 4.6). As can been seen in figure 4.6a the banding pattern is virtually identical between isolations. This is confirmed by the histogram representation of bands generated by ImageJ (Figure 4.6b). Both methods of visualization show the major protein component to be approximately 34kDa. Based on the protein size, and established gonococcal outer membrane protein literature, this band corresponds to the porin, P1B (Swanson, 1981; Blake and Gotschlich, 1982; Wetzler et al., 1988). In addition to demonstrating inter-batch consistence of protein components, figure 4.7 shows that the protein composition of wild-type nOMVs and Δ*rmp* nOMVs are extremely similar outside the band seen at approximately 28kDa which corresponds to RMP's known molecular weight (Lytton and Blake, 1986).

These results show that Δ*rmp* nOMVs not only have structural and morphological consistency but their protein antigen profile is extremely consistent between isolations as well. This is an important characteristic in a potential vaccine antigen since it will limit vaccine batch related variation in the induced immunity by providing the same population of potential immunogens each time.



Lane	Content
1	Standard protein ladder
2	10µg **Δ**rmp GC extract
3	10µg **Δ**rmp nOMV #1
4	10µg **Δ**rmp nOMV #2
5	10µg **Δ**rmp nOMV #3
6	5µg wt nOMV

Figure 4.7: The protein composition of nOMVs is not significantly altered by the exclusion of RMP. Protein extracted from Δ*rmp Neisseria gonorrhoeae* (Lane 2; 10µg), 3 isolations of Δ*rmp* nOMVs (Lanes 3, 4, and 5; 10µg) and wild type nOMVs (Lane 6; 5µg) were separated by denaturing PAGE and visualized with coomassie blue stain. Protein band thought to correspond to RMP (based on weight) is indicated.

Gonococcal *rmp* deficient nOMVs contain bioactive pathogen associated molecular patterns

The potential ability for OMVs to function as their own adjuvant in a vaccine preparation has already been discussed. Briefly, this capability could allow for the induction of a more complex immune response because it would allow for the exclusion of the largely immunologically unidimensional adjuvants that we have at our disposal. The immune stimulatory profile of nOMVs is extremely complex. Because they are derived from bacteria themselves, vesicles have been shown to carry a diverse array of known pattern recognition receptor (PRR) agonists like peptidylglycan (Kaparakis et al.,

2010), nucleic acids (Koeppen et al., 2016), lipoproteins (Wetzler, 2010; van de Waterbeemd et al., 2013), and lipopolysaccharides (van de Waterbeemd et al., 2010). Most of these structures have been shown to be present in GC nOMVs (Kaparakis et al., 2010; Zielke et al., 2014; Vanaja et al., 2016) and would contribute to the particle's overall immunostimulatory profile. These PAMPs additionally are likely to be particularly potent as nano-structure associated PAMPs have been shown to induce more efficient antigen uptake, activation of immune cells and induction of an adaptive immune response than their components in their soluble form (Wetzler et al., 1992b; Zhu et al., 2005; Kaparakis et al., 2010; Nicolete et al., 2011).

Based on both literature and our studies presented here, we focused on two specific PAMPs due to their large quantities in the GC outer membranes and likely nOMVs: P1B and Lipooligosaccharide (LOS). This bacterial porin is, by quantity, the primary outer membrane protein in pathogenic *Neisseria* species and was shown to be present at high concentrations in our nOMVs (Figure 4.6a). This protein is a well characterized TLR1/2 heterodimer agonist capable of inducing a strong adaptive immune response to itself without an exogenous adjuvant (Blake et al., 1989; Wetzler et al., 1992b; Zhu et al., 2005; Massari et al., 2006; Oliveira-Nascimento et al., 2012). The second PAMP, lipo-oligosaccharide (LOS – similar to gram-negative lipopolysaccharide [LPS], but with a shorter side chain), is a strong TLR4 agonist. The presence of LOS in the Δ*rmp* nOMVs was confirmed by silver stain (Figure 4.8).

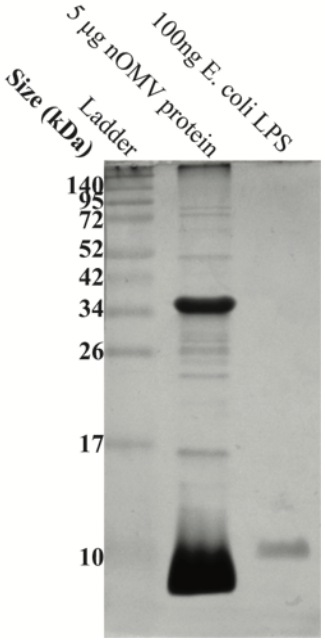


Figure 4.8: Natural vesicles released from Δ*rmp* strain GC contain a large quantity of LOS. Structures from 5µg nOMV (associated protein) were separated by denaturing PAGE. One-hundred nanograms of *E. coli* derived LPS was run in an adjacent well as a positive control. Protein and lipopolysaccharides were visualized by silver stain as described in Chapter 2.

It is evident from the silver stain in Figure 4.8 that there is a large quantity of LOS associated with Δ*rmp* nOMVs. A strikingly larger amount of staining is seen in the ~10kDa region where neisserial LOS is known to be found than what is seen for the 100ng *E. coli* LPS control. While this could potentially be concerning due to the toxic effect of high quantities of LPS, we first evaluated whether the large quantity of endotoxin corresponded to a high bioactivity. The ability of P1B and LOS as part of RMP-deficient nOMVs to bind and activate their corresponding PRRs was determined using human embryonic kidney (HEK) cells transfected with the genes expressing their respective TLRs. Three separate cell lines were used that had been transformed with a genetic construct containing either TLR1/TLR2, TLR4, or a stretch of non-coding DNA as a control. Since HEK cells do not naturally express TLRs, introduction of these constructs allows us to quantify the ability of a sample to act as an agonist of only the inserted PRR (by measuring induced cytokine production).

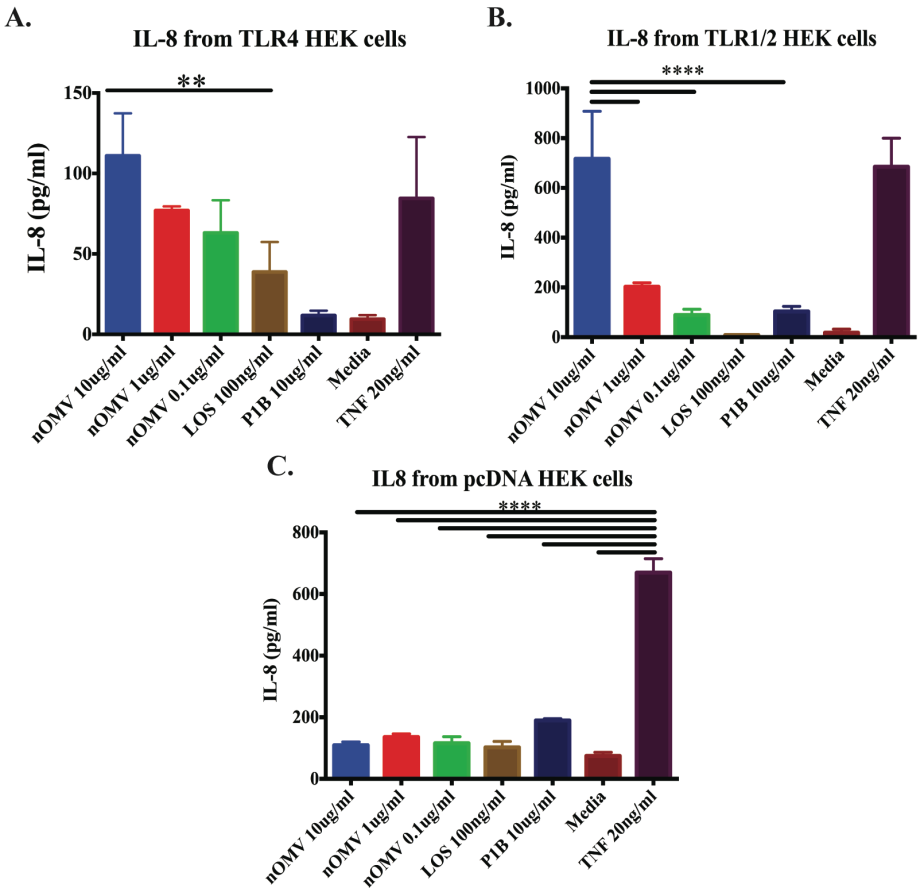


Figure 4.9: Δ*rmp* nOMVs bind to and strongly activate TLR1/2 and TLR4 signaling pathways. PRR activation was indirectly measured by secretion of IL-8 by HEK cells overexpressing TLR4 (**A.**), TLR1/TLR2 (**B.**) or control cells after a 12 hour stimulation with 10, 1 or 0.1 μg nOMVs, 100ng LOS, 10ug P1B, or 20ng TNF-α. Histogram shows mean with standard deviation, significance was calculated by one way ANOVA between each condition. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ by ANOVA. Data was analyzed and graphs were generated in Prism. Supernatant IL-8 levels were measured by ELISA (BD OptEIA Cat#555244).

As figure 4.9 shows, RMP-deficient nOMVs were able to bind and activate both TLR1/2 and TLR4. The HEK cells that were transfected with non-coding DNA were only activated by the non-TLR simulant, TNF-α (Figure 4.9c). This allows us to interpret any observed induction of IL-8 in our two TLR-transfected cell lines as the direct result of TLR agonism. In both TLR4 and TLR2/1 cells (Figure 4.9a and b respectively), there

was an nOMV dose dependent induction of Il-8. The high dose, 10μg nOMV was able to induce the greatest observed levels of Il-8 through both of the interrogated TLR pathways. For the TLR4 HEK cell stimulation, all three nOMV doses induced significant ($p<0.05$) levels of IL-8 (as determined by one way ANOVA against media). The high dose induced significantly higher levels than the 100ng LOS positive control. As expected, in the absence of TLR1/2, P1B did not induce Il-8 in the TLR4 HEK cells. These results suggest that even at 0.1μg/ml, nOMVs are able to induce non-inferior TLR4 activation as the standard dose of LOS/LPS we utilize as a vaccine adjuvant (Platt et al., 2013; Reiser et al., 2017). High dose nOMVs were able to powerfully activate TLR1/2, inducing over 7-fold greater levels of Il-8 than the standard 10μg adjuvant dose of P1B (Figure 4.9b). Both of the lower doses of nOMVs induced non-significant IL-8 production from the TLR1/2 transfected HEK cells. These results suggest that activation of a poly-PRR agonist adjuvant mechanism will require nOMV dosing towards the higher end of the tested range.

Gonococcal *rmp* deficient nOMVs are stable over long periods of storage

In addition to the successful induction of immunity, practical characteristics of a vaccine can also greatly impact its success. Stability is an important characteristic in the evaluation of a potential vaccine antigen and is one of the factors that greatly impacts the cost of a vaccine, due to the effect on ease of its storage, handling and delivery (Plotkin et al., 2017). Detergent extracted Nm OMVs (dOMVs) have been shown to be remarkably stable maintaining structural and antigenic durability throughout long periods of storage,

freezing and thawing as well as lyophilization (Arigita et al., 2004). This stability of the Nm dOMV vaccine has aided its adoption and delivery worldwide (Granoff, 2010). We determined that GC nOMVs demonstrate the same structural stability.

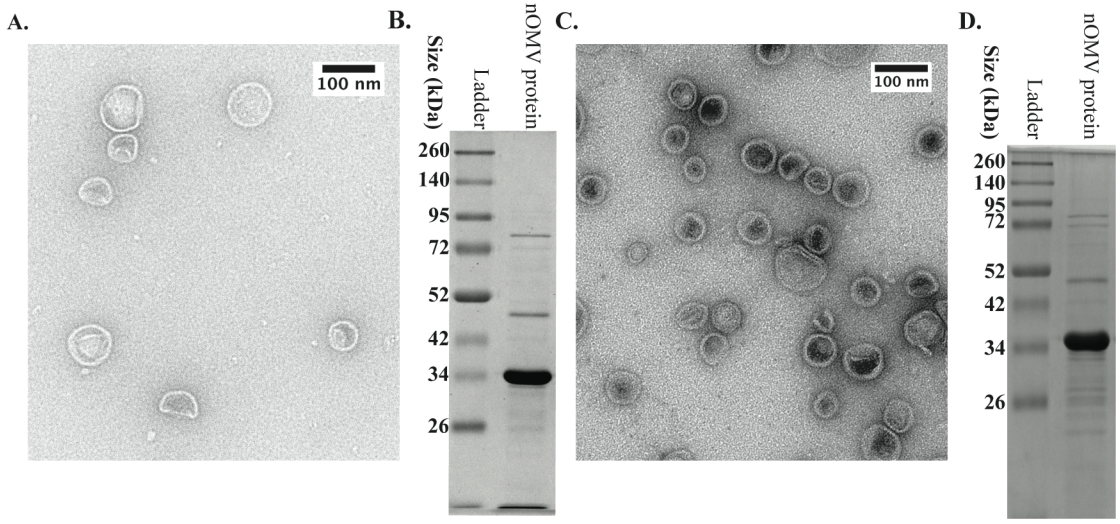


Figure 4.10: nOMVs lacking RMP, display stability for up to 1 year in standard 4^{O}C storage conditions. Vesicles were isolated from a Δ*rmp* strain of GC and evaluated at 2 weeks (**A.** and **B.**) and 12 months (**C.** and **D.**) after isolation for morphology by UA negative stain TEM (**A.** and **C.**) and protein composition by PAGE and Coomassie blue staining (**B.** and **D.**). PAGE was run using 5μg of nOMV associated protein (in indicted lanes). Images were processed and scale bars generated in ImageJ. Contrast differences are the result of different image capture methods (CCD camera versus film based Plate camera).

As seen in figure 4.8, vesicles had the same morphology following 1 year of storage in sterile conditions at 4^{o}C (Figure 4.10c) as they demonstrated right after isolation (Figure 4.10a). Similarly, the protein banding pattern appears relatively unchanged following the same storage period (Figures 4.10b and d). These results reflect what has been previously reported regarding the Nm dOMVs (Arigita et al., 2004) and suggests that these GC nOMVs might display the same characteristics that have made these vaccines a global public health success (Granoff, 2010; Plotkin et al., 2017). With

these encouraging *in vitro* results, we then started to investigate the *in vivo* response to Δ*rmp* GC nOMV as a vaccine candidate.

Gonococcal nOMVs lacking RMP induce a dose dependent, systemic and mucosal, antibody response to intranasal or subcutaneous immunization

As mentioned previously, the few gonococcal OMV vaccine studies that have been published utilized chemical- or detergent-extracted vesicles from WT GC strains, which are known to differ from the nOMVs derived from RMP minus GC that we are using. Additionally, all previous studies have investigated vaccine preparations that included exogenous adjuvants. As shown in chapter 4, Δ*rmp* nOMVs are potently immunostimulatory and likely do not need an exogenous adjuvant in order to be highly immunogenic. Since previous work could not be used to inform the optimization of our vaccine, we decided to examine the anti-nOMV response to a variety of vaccine dosages. Additionally, we believe that route of immunization will significantly impact the ability of the vaccine to protect the FGT, therefore we compared immune responses after intranasal and subcutaneous immunization. To determine dose and route effects, 38 wild-type C57/BL6 female mice were divided into 2 groups based on route of immunization, subcutaneous (SC) or intranasal (IN). The 19 mice in each route group were then divided as follows: 4 mice received PBS, 5 mice received 0.1μg nOMVs, 5 mice received 1μg nOMV, and 5 mice received 10μg nOMV. All mice received 3 RMP-deficient nOMV vaccines (or PBS) of a given dose, via injection or intranasal deposition, each dose

separated by 2 weeks. Sera and vaginal secretions were collected the day before the first injection and 2 weeks following the final injection.

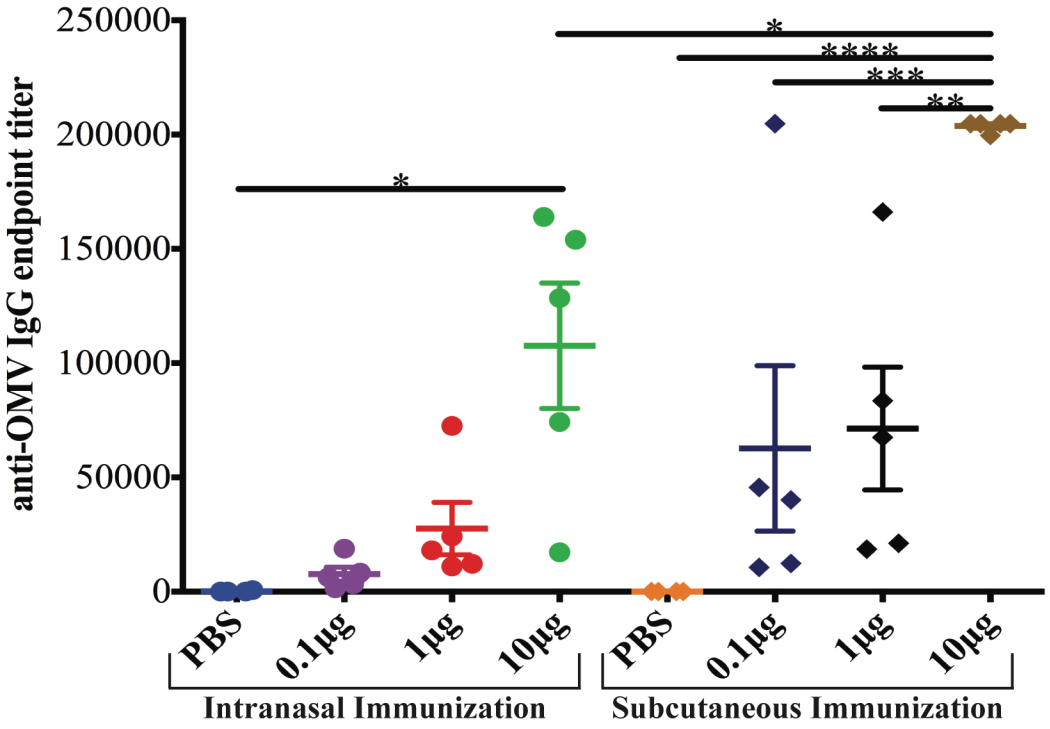


Figure 4.11: Intranasal and subcutaneous immunization with *Neisseria gonorrhoeae* Δ*rmp* nOMVs induces high titers of serum anti-nOMV IgG in a dose dependent pattern. Mice (N=5 for each nOMV vaccine group, N=4 for each PBS control group) received either nOMVs or PBS via the indicated route three times separated each time by a period of 2 weeks. Sera was collected 2 weeks following final exposure. Serum titers of vaccine induced nOMV specific IgG were evaluated by nOMV ELISA. Data was analyzed by one way ANOVA and graphed in Prism. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ by ANOVA

The immunogenicity of each vaccine preparation was measured by nOMV ELISA to determine titers of IgG able to bind the vaccine antigen, in serum taken 2 weeks after the final vaccine. We first examined serum Ig, despite our belief that a mucosal response will be key to protection, because vaginal Ig concentrations can be low and hard to measure; and the secreted IgG profile is thought to reflect the systemic IgG profile (Li et al., 2011). Figure 4.11 shows that nOMVs are able to induce very high serum titers of

nOMV-specific IgG, particularly when delivered subcutaneously. The mice that received SC immunizations all showed significant titers of nOMV-specific IgG that increased in magnitude in a dose dependent pattern. This particularly strong response following SC immunization is not surprising as SC is a parenteral vaccination that is known to efficiently induce systemic immunity. Intranasal immunization induced significant serum titers of anti-nOMV IgG only with the highest dose vaccine. This significant response induced greater titers than that of the SC 1μg vaccine but significantly less than the SC 10μg dose. The strong induction of systemic immunity following IN immunization was not necessarily expected and may indicate a "spill over" from a particularly strong mucosal response.

The mucosal response to nOMV immunization was evaluated by nOMV ELISA to determine IgG titers present in vaginal secretions 2 weeks after the final booster immunization. As previously mentioned, the low concentration of Ig in vaginal secretions makes it hard to absolutely quantify, so results are presented as the Optical Density absorbance of 405nm light (OD_{405} at 1:6400 titer) in the ELISA. In comparing the two routes of immunization, mice receiving the SC vaccines were more likely to have measurable anti-nOMV IgG in their vaginal secretions following 3 immunizations as compared to IN immunized mice (figure 4.12). This is somewhat surprising considering the body of literature suggesting a strong mucosal response is best induced by mucosal exposure to an antigen. Despite this, only two mice, both from the high dose group, showed measurable anti-nOMV IgG from the IN vaccinated mice. The 10μg SC vaccine had the best rate of inducing measurable antigen-specific IgG with 4 of 5 mice. The

greatest nOMV-specific vaginal IgG response was seen in a mouse from the 0.1μg SC immunization group. This mouse was the only one from its treatment group to have measurable anti-nOMV IgG in vaginal samples. This response is very surprising and a clear outlier within its treatment group. Interestingly, this mouse was the same mouse that demonstrated extremely high serum IgG titers (Figure 4.11). While this result is likely not indicative of the standard immune response to the 0.1μg SC vaccination protocol, it does confirm that a sufficiently robust systemic response can translate to a strong mucosal response. This means that while a mucosally directed immunity is preferred, if that proves unattainable, we may still be able to induce a strong FGT antibody response by inducing a powerful systemic response.

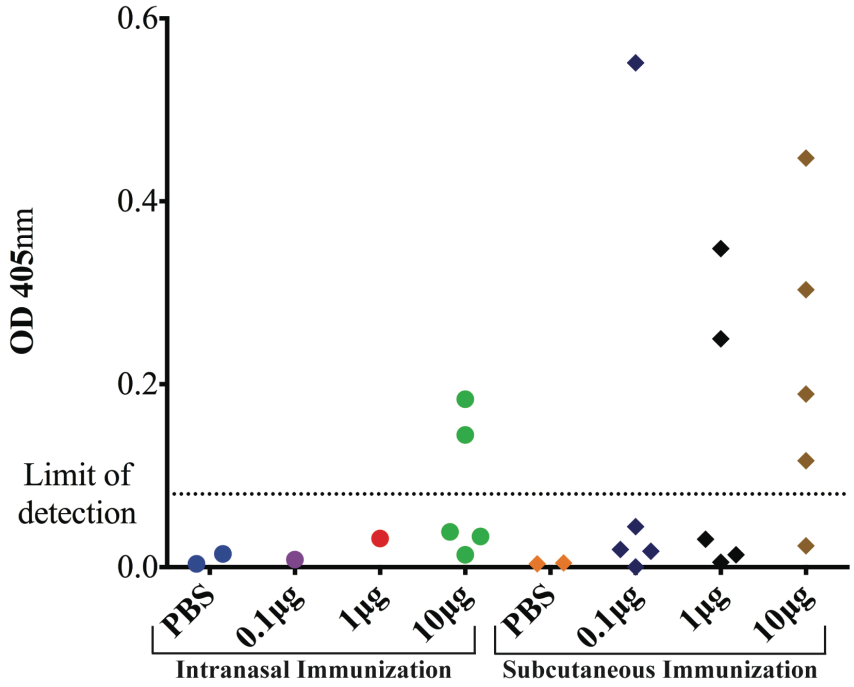


Figure 4.12: Mice receiving subcutaneous nOMV vaccines are more likely than those receiving intranasal vaccines to have measurable anti-nOMV IgG in vaginal secretions after 3 vaccinations. Mice (N=5 for each nOMV vaccine group, N=4 for each PBS control group) received either nOMVs or PBS via the indicated route three times separated each time by a period of 2 weeks. Vaginal secretions were collected 2 weeks following final exposure. Graph depicts levels of vaccine-induced nOMV-specific IgG in vaginal secretions as measured by nOMV ELISA. No significant differences between groups were found. Data was analyzed by one way ANOVA and graphed in Prism

The poly-immunogenic response to immunization with RMP deficient nOMVs is dose and route dependent

One of the unique characteristics of nOMVs as vaccine antigens is their poly-antigenic composition. These vesicles offer dozens of potential immunogens to the host immune system (Zielke et al., 2014). It is our thought that a diverse immune response against several distinct gonococcal immunogens, could place overwhelming stress on the pathogen. We demonstrated in chapter 4 that our RMP-deficient nOMVs contain multiple

protein antigens, however we would expect that not all of them would be strongly immunogenic. To determine which of the nOMV antigens were immunogenic, we ran immunoblots where immobilized nOMV associated antigens were probed with immune sera from each of our treatment groups.

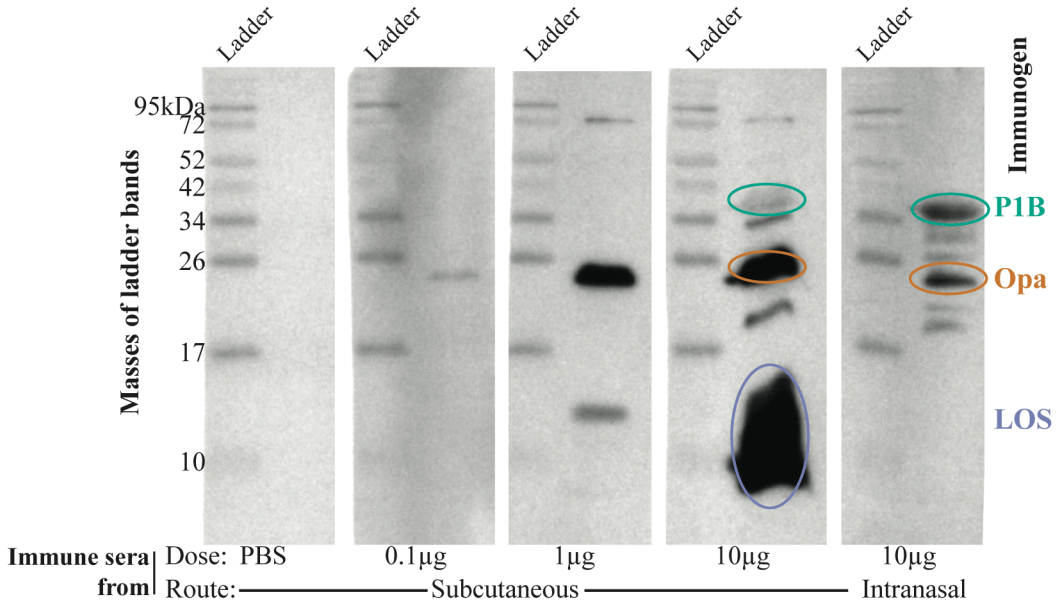


Figure 4.13: The humoral response to nOMVs recognizes multiple immunogens in a dose and route dependent pattern. PAGE was used to separate standard proteins (first lane of each membrane) and structures in 10µg of Δ*rmp* nOMVs (second lane of each membrane) by molecular weight. Structures were then transferred to PVDF membrane and each membrane was individually probed with pooled immune sera (1:1000 concentration in 10% non-fat dry milk in PBS solution) from all mice in a given treatment group. The treatment group from which the probing sera was taken is indicated above each membrane. Bound vaccine-induced nOMV specific IgG was visualized using Horseradish peroxidase conjugated horse anti-mouse IgG (Cell signaling, Cat#7076S) and ECL chemiluminescent solution (GE Healthcare, Cat#RPN2232) and captured by Amersham Hyperfilm™ (GE Healthcare, Cat#28906837). Film was exposed for 60 seconds. The suspected identities, based on molecular weight, of three strong immunogens are indicated to the right of the figure and are color matched to the highlighting oval.

Since only the subcutaneously immunized and high dose intranasally immunized mice had statistically significant levels of anti-nOMV IgG, we only examined the pattern

of RMP-deficient nOMV antigen immunogenicity in those mice. The pattern of immunogen recognition, as shown by figure 4.13, was highly dependent not only on the vaccine dose given, but the route of immunization. All subcutaneously immunized mice developed a response against the same ~26kDa protein. For the 0.1μg subcutaneously immunized mice this was the only immunogen recognized. This same protein was the primary immunogen in the 1μg subcutaneous vaccine group as well as the primary protein antigen in the high dose SC group. While this protein cannot be positively identified here, its size suggests it may be an Opa protein (Swanson, 1978). While the low dose SC group was limited to this one immunogen, both the intermediate and high dose SC vaccine groups demonstrated the poly-immunogenic response that we expected to see from nOMV immunizations.

The number of antigenic targets was directly related to dosage with the intermediate vaccine inducing a response against 3 antigens and the high dose have a complex response against at least 7 different immunogens. At least 2 immunogens were shared between the two groups, the ~26kDa immunogen and a high molecular weight structure that has a mass around 72kDa. Surprisingly of the subcutaneous vaccine groups, the major outer membrane protein P1B, which is highly immunogenic in its soluble form (Zhu et al., 2005), was only immunogenic (band around 34kDa) in the high dose group. The humoral response induced in the high dose SC group was dominated not by a protein antigen, but by LOS. The extremely large smear seen in the 10μg SC immunoblot appears to be caused by immune recognition of a small (~10kDa) structure that would correspond with gonococcal LOS. The majority of the immunogen recognition by the

10μg SC immune-sera was directed against LOS and the Opa-like protein. The other immunogens demonstrated significantly weaker signal in the immunoblot.

The high dose intranasal immunization group displayed a very different response than what was seen in SC mice. At least 6 separate immunogens were recognized by the immune sera. The two primary immunogens were the ~26kDa protein and a ~34kDa protein that is most likely P1B. What makes the 10μg IN induced response different was that the minor immunogens were still recognized relatively strongly as compared to the minor immunogens of the 1 and 10μg SC mice. Additionally, the immunogenicity of LOS appears to be entirely abrogated by IN immunization. This may be a beneficial characteristic since gonococcal LOS can undergo significant modification depending on environmental conditions (Wetzler et al., 1992a; Yang and Gotschlich, 1996; Lewis et al., 2015) which could allow the bacteria to avoid an anti-LOS response induced by the nOMVs.

These studies were designed to help determine the optimal dose and route for nOMV immunization. We were able to determine that both of these variables have significant impact on the magnitude and targets of the induced humoral response. Our results show a dose dependent pattern in the magnitude, strength of immunogen recognition and number of immunogens in the RMP-deficient nOMV induced anti-nOMV IgG response. Both 10μg vaccines, intranasal and subcutaneous, were able to induce a strong, poly-antigenic anti-nOMV immune response like what we hoped for. This is valuable information in the evaluation of the vaccine, however it is recognition and binding to the pathogen that is needed to possibly confer protection against infection.

Since the 10μg vaccine induced anti-nOMV responses differed in their specific immunogen profile by vaccine route, we chose to move forward with the evaluation of both IN and SC 10μg vaccines.

nOMVs induce a strong anti-gonococcal humoral response

One of the inherent benefits of an nOMV vaccine is thought to be the concentration of outer membrane structures in their native confirmation (van der Pol et al., 2015). This should result in the induction and subsequent boosting of immune responses directed towards these externally exposed antigens. Such a response would be expected to recognize the same immunogens in the source pathogen upon exposure. However, due to the lack of rigorous proteomic structural studies of nOMVs, it is possible that during the process of vesicle formation there are modifications made to its antigenic cargo that render the induced immune response unable to bind the cellular form of the antigen. Using the optimized 10μg dose RMP-deficient nOMV vaccine, we investigated the ability of the induced humoral response to bind gonococci.

Through our previous studies we have shown that IN and SC immunization with RMP-deficient nOMVs results in strong but distinct immune responses. The subcutaneous immunizations were able to induce greater titers of anti-nOMV IgG in the sera and vaginal secretion than IN, however the SC response was dominated by an anti-LOS response that is potentially non-protective. In contrast, the IN vaccine induced a diverse and more balanced response to an array of immunogens. As previously mentioned, these two vaccines each present unique benefits and drawbacks and will be

evaluated in tandem. Additionally, we decided to examine the response to a mixed route vaccine. There is some precedence in the literature that suggests a vaccine that is introduced systemically and mucosally in the same animal, can induce an immunological profile that contains characteristics of both a SC and IN only vaccine (Plante et al., 2000). To test that with our RMP-deficient nOMVs, we included a group of mice that were immunized with 10μg nOMV in an intranasal-subcutaneous-intranasal (IN-SC-IN) pattern for the 3-injection protocol.

Using the same protocol as our previous study, 3 injections each separated by 2 weeks, thirty 9-week old wild-type female C57/BL6 mice were separated into 6 different experimental groups as follows: 3 mice SC 3xPBS, 10 mice 3xSC 10μg nOMV, 3 mice 3xIN PBS, 10 mice 3xIN 10μg nOMV, and 4 mice 10μg IN-SC-IN. Each of the two groups with 10 mice were separated into two cages each with 5 mice. Sera was collected from each mouse prior to each immunization and 2 weeks after the final injection. Vaginal secretions were collected prior to the first and 2 weeks after the final immunization. During the study, 3 mice from the subcutaneous 10μg vaccine group developed ulcerative lesions on their flank following the first immunization and had to be sacrificed on day 18 without receiving additional vaccines. All other mice remained healthy and completed the full protocol. To assess the ability of the anti-nOMV responses to the various vaccination protocols to bind whole gonococci, we measured binding of serum IgG from mice that received all 3 vaccinations in a whole cell ELISA.

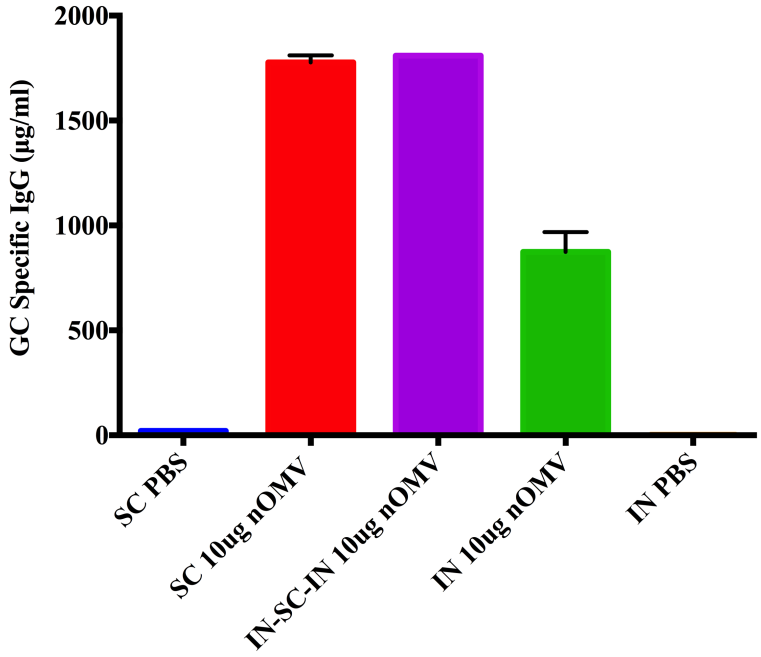


Figure 4.14: Subcutaneous and mixed route nOMV vaccines induce high levels of GC-specific IgG after 3 immunizations. Mice (N=10 for IN nOMV vaccine group, N=7 for SC nOMV vaccine group, N=4 for IN-SC-IN group, N=3 for each PBS control group) received either nOMVs or PBS via the indicated route three times separated each time by a period of 2 weeks. Sera were collected 2 weeks following final exposure. Graph depicts concentrations of vaccine-induced GC-specific IgG as measured by whole bacteria ELISA. Sera from mice in a single cage was pooled in equal volume and evaluated for GC-specific IgG. Bars show mean and SEM for calculated GC-specific IgG concentrations in sera pooled from all animals in a treatment group or averages for treatment groups split into two cages. Plates were coated with wild-type MS11 strain *Neisseria gonorrhoeae* at a density of $5x10^6$ gonococci/well. Data was analyzed and graphed in Prism.

Following 3 immunizations, there were extremely high serum levels of IgG able to recognize and bind whole gonococci (Figure 4.14). Similar to the anti-nOMV response, the SC immunization induced greater levels of GC-specific serum IgG than the IN immunization. Since the other groups had only one value to observe, statistical comparisons cannot be run, however it appears that the mixed immunization protocol induced non-inferior levels of GC-specific IgG as compared to SC and greater levels than the IN immunization. This suggests that this mixed immunization protocol may be able to

at least recapitulate the strong systemic immune response of the purely SC vaccination if not the mucosal elements of the IN vaccine. With the development of inflammatory lesions and early sacrifice of three 10μg SC mice, we were able to examine how the primary vaccine response develops beyond the point where we usually induce a secondary response.

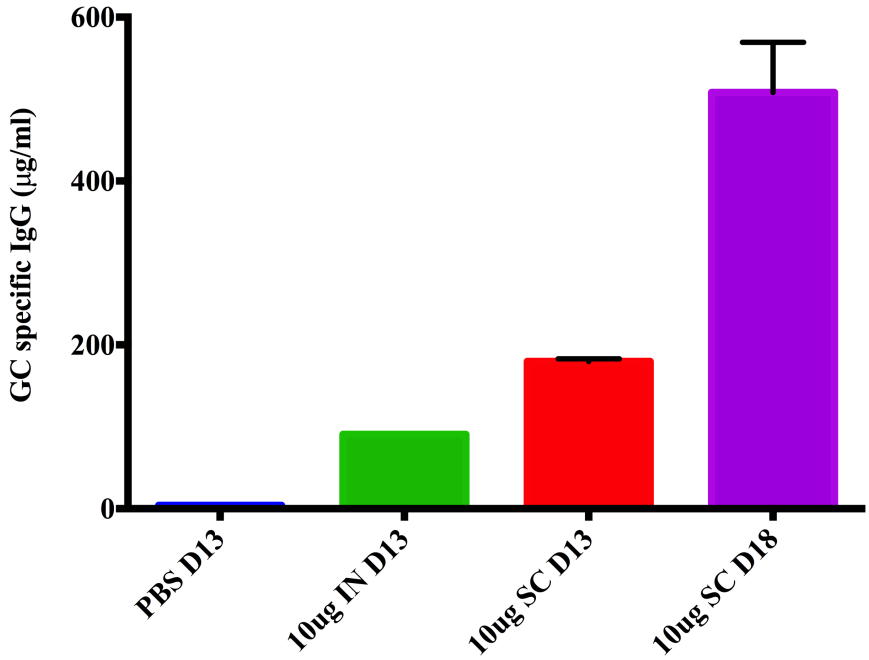


Figure 4.15: Subcutaneous immunization with nOMVs induces a robust anti-GC response even after only 1 immunization. Mice (N=1 PBS D13 and 10μg IN D13, N=2 10μg SC D13, N=3 10μg SC D18) were exposed to the indicated vaccine or PBS treatment via the indicated route (IN versus SC). On either day 13 or day 18 (as indicated in the figure) following exposur, sera was collected and evaluated for concentration of GC-specific IgG as measured by whole GC ELISA. Bars show mean and in the case of multiple samples, SEM. Plates were coated with wild-type MS11 strain *Neisseria gonorrhoeae* at a density of $5x10^6$ gonococci/well. Data was analyzed and graphed in Prism.

We were very surprised to see such high levels of GC-specific IgG in the SC and IN immunized mice after only 1 vaccine. Even more surprisingly, it appears that the adaptive response to the vaccine was still building rather than resolving at day 13 as the

levels of GC specific IgG were even higher at day 18 (Figure 4.15). These results suggest that the vaccine induced humoral response is extremely robust without any secondary exposure. This may mean a single dose of nOMV vaccine could induce an adequate response, at least in magnitude, for protection.

Discussion

The development of an anti-gonococcal vaccine is a critical public health need as we enter an era of antimicrobial-resistant *Neisseria gonorrhoeae*. While there have been intermittent explorations of gonococcal outer membrane vesicles as vaccine antigens, none have utilized the necessary Δ*rmp* source strain to prevent a host-detrimental anti-RMP response, and none have focused specifically on the naturally released vesicles (nOMVs). In the studies presented here, we report the first isolation, and subsequent preliminary characterization of Δ*rmp* GC nOMV structure, protein composition, and immunostimulatory capabilities that we are aware of. The findings of these studies suggest that these vesicles can be isolated with a consistent profile between batches and are highly immunostimulatory. Additionally, they are remarkably stable in their composition over long periods of storage.

Through the work of Zielke and colleagues (Zielke et al., 2014), we know that nOMVs from any given strain, contain a vast array of antigens. They identified known vaccine candidate antigens including P1B, pilis components, iron acquisition molecules, drug efflux pumps and several others (Zielke et al., 2014). Although a detailed characterization of the Δ*rmp* nOMV has not yet been completed, we did show by PAGE

that their overall proteome did not appear to significantly differ from that of wild type nOMVs. We can then assume that some of these vaccine candidate proteins are present in our RMP-deficient nOMVs as well. While these *in vitro* findings are encouraging, the *in vivo* studies presented here offer the strongest evidence for the potential of *Neisseria gonorrhoeae* Δ*rmp* nOMVs as a promising anti-gonococcal vaccine.

We demonstrated that these particles can induce an extremely robust systemic humoral response that not only recognizes the vaccine antigen but binds the whole gonococci as well. Additionally, we showed that the anti-nOMV humoral response demonstrates the poly-antigenic nature that we believe may be advantageous in inducing protective immunity against GC. Our studies did not, unfortunately seem to induce the strong mucosal immunity that we hoped. We showed that some mice, following 3 nOMV immunizations, had measureable anti-nOMV IgG in their vaginal secretions. Most of these mice received subcutaneous immunizations rather than intranasal. This suggests that the route of vaccine administration may not be as important to inducing mucosal tissue immunity as we had hypothesized. In fact, the greatest measureable level of vaginal anti-nOMV IgG was from a low dose SC vaccine hyper-responder. This would suggest that a systemic immune directed vaccine may be superior in the induction of vaginal IgG.

The results of our initial optimization experiment are even more interesting in the context of our initial observations on the impact of mixed-route immunization protocols. In the initial experiment, IN only immunization induced not only a weak mucosal response but a weak systemic one as well. In the evaluation of the IN-SC-IN protocol we

showed that by including just a single SC immunization we could boost the magnitude of the systemic anti-nOMV response to the level of the SC only mice. If the weak anti-nOMV response observed in the IN-only mice, is due to limited immune engagement when delivered via the nasopharynx, a systemic boost could overcome this obstacle. A mucosally influenced primary immune response would be preferentially expanded upon secondary exposure despite being via SC, and finally reinforced with a third and final IN vaccine resulting in strong systemic immunity directed towards mucosally active immunogens. This mucosal prime, systemic boost and mucosal finish approach is similar to a "prime and pull" method that has been investigated in the field of HIV vaccinology with moderate success (Tregoning et al., 2013). While the studies presented here showing the strong poly-antigenic immunogenicity of Δ*rmp* GC nOMVs are encouraging for the vaccine potential of an nOMV GC vaccine, further study of the anti-nOMV immune response is needed; particularly with respect to determining protective mechanisms.

Limitations of presented studies

A major limitation of these studies is the lack of regulation of antigens included in the nOMVs. While we show, via coomassie stained PAGE, that the protein banding patterns are consistent between isolations, this is far from definitive proof of antigenic consistency. The gonococcus is known to be extremely sensitive to changes in the environment and that these changes directly impact the expression profile of its outer membrane proteins. Even the slightest drop in atmospheric O_2 leads to the insertion of anaerobic metabolism related membrane proteins into the outer lipid bilayer (Clark et al.,

1987) and these proteins can be included in released nOMVs (Zielke et al., 2014). A similar effect has been described during growth in iron poor media (West and Sparling, 1985). Of course this potential source of vesicle heterogeneity is minor compared to the stochastic expression pattern of the Opa proteins (Muralidharan et al., 1987). We have already mentioned the potential for LOS modification leading to alteration of exposed epitopes. The goal of this vaccine is to induce a poly-antigenic response, however the protective potential of this response is undermined if the majority of the immunogens represent transiently expressed proteins or modified forms of proteins. These limitations can be addressed first, by the systematic characterization of vesicle associated antigens by liquid chromatography mass-spectroscopy (LC-MS). Protein sequencing as well as LOS analysis by LC-MC are both well-established quantitative processes that would allow us to determine antigenic composition of Δ*rmp* nOMVs as well as quantify any variation between batches.

With this knowledge, we would be able to identify whether there are potentially unproductive immunogens in the vesicles. For some of these antigens, we might be able to limit their impact through genetic modification of the Δ*rmp* strain of GC. In the case of the Opa proteins, the production of "Opa-locked" strains of GC has been previously achieved (Ritter and Genco, 2018). These strains are manipulated so that Opa expression is limited to a single isoform, or entirely repressed. In the case of LOS, there have been extensive studies on the genetic manipulation of neisserial LOS in order to prevent the full construction of the endotoxin (van der Ley et al., 2001). This approach sacrifices the

LOS-mediated immunostimulatory capability of the nOMVs however this is only a portion of their endogenous adjuvant activity.

A second limitation to our studies, was incomplete assessment of the mucosal antibody responses. The goal of our potential vaccine, and any ideal anti-gonococcal vaccine, is the formation of long lived, broadly reactive, mucosally active immunological memory. The cornerstone of such a protective immunity is likely to be mucosally secreted antibodies. While we presented some data regarding the detection of nOMV-specific IgG in vaginal secretions, we were unable to reliably quantify the ELISA results. An equivalent IgA ELISA was run but the assay had a near total lack of signal (Figure 4.16).

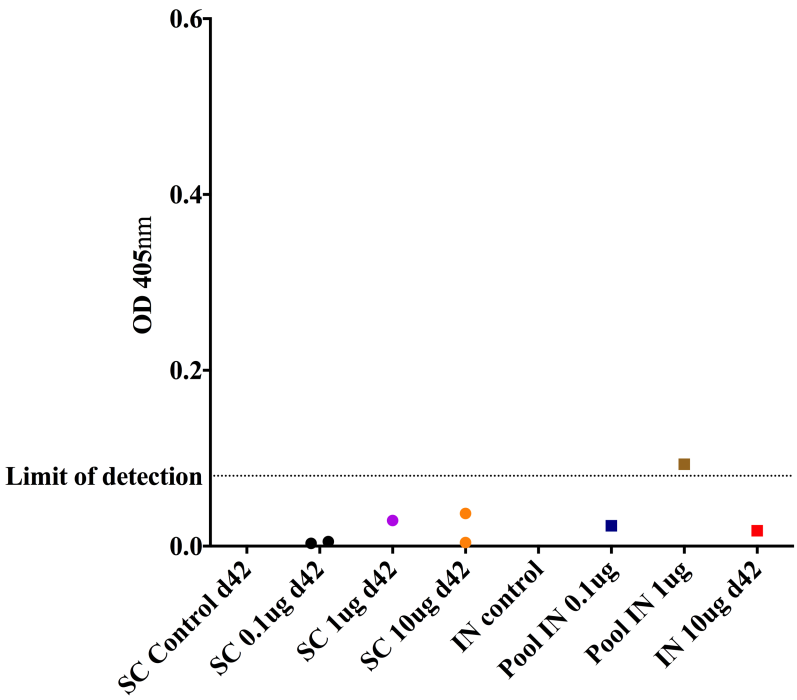


Figure 4.16: Vaginal nOMV specific IgA could not be detected in most samples. Mice (N=5 for each nOMV vaccine group, N=4 for each PBS control group) received either nOMVs or PBS via the indicated route three times, separated each time by a period of 2 weeks. Vaginal secretions were collected 2 weeks following final exposure. Graph depicts levels of vaccine-induced nOMV-specific IgA in vaginal secretions 2 weeks after the final immunization, as measured by nOMV ELISA (measured by OD405 absorbance caused by PNPP activation by AP conjugated rat-anti-mouse-IgA secondary antibody; Southern Biotech Cat# 1165-04). Samples from mice that showed no significant antigen specific humoral response in sera were pooled in equal volume while individual samples were evaluated for those treatment groups that showed significant antigen-specific sera IgG. Data was graphed in Prism. Limit of detection was provided by Southern Biotech.

The low or absent levels of antigen-specific antibodies in vaginal secretion may be due to protocol and assay insensitivity rather than true absence of a response. The process of sampling vaginal secretion by vaginal wash mandates the several hundred-fold dilution of secretions by the PBS wash solution. This takes the already relatively low concentrations of antibodies, compared to sera (Usala et al., 1989), and dilutes them to the point where they become exceeding difficult to accurately measure. There are

methods of collection that limit the dilution of vaginal secretions, like the extended placement of a surgical sponge into the vaginal os. This method requires more time than a simple vaginal wash and quickly becomes impractical with large numbers of animals like what is needed in studies of vaccine induced host responses. There have been published methods that may help with the isolation and measurement of vaginally secreted Ig including the repeated extraction of proteins from vaginal mucus. Our failure to extract Ig from isolated mucus, instead focusing on Ig within the liquid phase of our wash samples likely negatively affected our ability to measure antigen specific Ig. The strong association of Ig with vaginal and cervicovaginal mucus is well documented (Fahrbach et al., 2013). The optimization of antigen specific Ig ELISAs using human genital secretions has been extensively studied (Donadoni et al., 2010) and these methods may help to increase the sensitivity of our murine assays. These alterations, along with utilizing a more sensitive assay like flow cytometry, Luminex assay or immunofluorescence may help us better evaluate and quantify the vaginal secretion of antigen specific IgG and IgA. As our work progresses and we are able to examine transgenic male mice and their response to GC infection, we may be able to start working towards the assessment of their mucosal immune response however there are major technical challenges with this approach.

The development of an ulcerative lesion on 3/10 mice vaccinated SC with 10μg nOMVs in one of our immunization studies is concerning. These particles were shown to be extremely immunostimulatory. We view this as a potential asset as it eliminates the need for an exogenous adjuvant however it does carry the risk of causing destructive

inflammation by over stimulation of PRRs. The incidence of this adverse reaction needs to be investigated and if found to occur reliably with nOMV injection, an alternate formulation is needed. The detoxification of OMVs for use as a vaccine is well described with the *Neisseria meningitidis* vaccine. In that vaccine, the LOS content was determined to be too great for safe use in humans, therefore the vaccine was designed around detergent extract dOMVs which have significantly less LOS (Granoff, 2010). Additionally, the adjuvant, alum, was included which is known to abrogate the toxic effect of LOS (Kool et al., 2012). While we believe the use of dOMVs would lead to a less protective immune responses to their significant cytoplasmic contaminants, the detergent extraction of pre-isolated nOMVs could reduce LOS levels while maintaining the nOMV protein profile.

Another limitation of these studies is that they were carried out in female wild-type C57/BL6 mice. The transgenic mice presented in chapter 1 and in which *in vivo* protection studies will be carried out, were constructed on an FvB genetic background. Our collaborators are currently backcrossing these mice onto the C57/BL6 background however they will still be genetically distinct from pure wild-type C57 mice. The strain dependent variability in immune response to immunizations is a phenomenon that the field of vaccinology has struggled with for decades (Eisenstein et al., 1984). It is likely that the immune response to nOMV immunization presented here, will differ from that of the transgenic mice, partially due to their distinct genetic backgrounds, but also by virtue of their transgenes. We believe that these vesicles contain Opa proteins that are conceivably able to interact with the hCEACAMs of the transgenic mice. The

implications of this are entirely unknown. Following engagement with hCEACAM3 on a neutrophil, the vesicles would likely induce activation of the cell. It is possible that this could result in greater innate system response that leads to a more robust adaptive response. However, it is just as likely that this could induce a damaging non-specific inflammatory response. One possible benefit would be related to antigen uptake in the IN immunizations. There have been no studies looking at CEACAM expression in the nasopharyngeal epithelia that we are aware of. If they are expressed, it is conceivable that nOMV Opa engagement of the receptors could increase antigen uptake following IN immunization.

Many of these limitations are inherent in early *in vivo* testing of novel vaccines. Without known mechanisms of protection, we are forced to make guided hypotheses on when, how, and where to look when evaluating the resulting immune response. As we move forward, we will refine our methods to overcome the technical problems limiting the evaluation of the mucosal response. We will be able to test the vaccine(s) in wild-type FvB and finally the transgenic lines themselves to evaluate the impact genetic background and hCEACAM expression has on the anti-nOMV response. We feel these limitations do not undermine our conclusions presented here, rather they show how much further there is to go as we try to understand these complex antigens and their *in vivo* activity.

Chapter 5: Implications and Future Directions

Fully drug resistant *Neisseria gonorrhoeae* represents a clear and present danger to global public health (CDC, 2012). With resistant strains already spreading and causing disease (Ohnishi et al., 2011), we are behind in the race to develop a solution to this threat. Although new-generation antibiotics have been described and are making their way through the regulatory process, these therapeutics, like every other anti-gonococcal antimicrobial before them, likely will be rendered useless with time (Unemo and Shafer, 2014). To adequately and definitively address this emerging "super-bug" a vaccine is needed. We feel that the studies presented in this body of work have moved the field closer to the realization of this goal.

In presenting novel models of gonorrhea we have expanded the toolbox that can be utilized by the gonococcal vaccine field at large to more rigorously test their formulations. This alone marks a remarkable step forward. Perhaps the greatest hurdle to the development of an anti-gonococcal vaccine is the lack of understanding the necessary mechanisms for protection. The previous model exhibited little infection induced pathology and therefore was a poor model to quantify the prevention of that pathology (Packiam et al., 2010). Now that we have a model of infection with robust pathology and characterized host responses, we may be more able to measure and investigate protective mechanisms. Once any amount of protection is described (by quicker bacterial clearance, increases in ID50, decreases in inflammatory pathology or other indirect measurements of protection), the reverse-engineering of that response to identify a correlate of immunity will provide the roadmap for the next evolution of anti-gonococcal vaccines.

We think that this initial hint towards a protective mechanism might have already been described. It was mentioned previously in this work that an as-of-yet uncharacterized partial protection against GC infection was identified in New Zealand during epidemiological studies of that country's recent nation-wide immunization program with the Nm group-B OMV vaccine (Petousis-Harris et al.). Although this protection is conservatively estimated to be only ~30% effective, this represents the first indication of any real-world protective immunity against GC. This immunity appears to result from an unintentional cross-reactive effect of the group B meningococcal outer membrane vesicle vaccine. Although there is some homogeneity between Nm and GC antigens, no such protective cross reactivity has been previously described despite the thorough investigation of several proteinaceous Nm vaccine antigens. This leads us to conclude that the protection is likely due to an unidentified antigen found in the OMVs. This is particularly exciting because this suggests that not only has a potentially protective antigen been found, but that a systemic intramuscular immunization is able to at least partially induce genitourinary protection.

The obvious question that arises from cross-reactive response to the Nm OMV vaccine is what sort of protection can be induced by a GC OMV? To answer this question, first new tools were required. For GC vaccines, unlike Nm, the outer membrane protein RMP must be excluded from preparation or else the induced response could result in decreased bactericidal activity, which may lead to increased susceptibility to infection rather than protection. Prior to the work presented here, no one had reported the isolation of OMVs lacking RMP. Not only did we successfully isolate vesicles that lacked RMP,

but we optimized a protocol that isolated nOMVs which are known to be enriched for outer membrane proteins and relatively free from cytoplasmic components (van de Waterbeemd et al., 2013; Zielke et al., 2014). The composition of Δ*rmp* nOMVs sets them up to be powerful inducers of anti-gonococcal responses against outer membrane structures. In fact, we showed here proof of concept results for the immunogenic potential for Δ*rmp* nOMVs.

Perhaps the most exciting part of the work that has been presented here, is that it represents early steps in a new direction. There will need to be extensive studies on the murine host response to GC infection in the context of hCEACAMs as well as other human restricted factors, like those that mediate bacterial iron acquisition and immune evasion. With greater advances in genetic manipulation of higher order mammals we hope to one day examine modeled GC infection in a mouse expressing all of these human factors. This would result in the creation of a true biological model of infection rather than a phenotypic model. Each of these studies will grow our understanding of how the immune system interacts with the gonococcus and how we might create an immunological memory that protects against it. Based on these results as well as a better understanding of the bacterial transcriptome during infection, we could curate the proteome of gonococcal nOMVs so that advantageous antigens are retained and extraneous ones excluded. This sort of genetic engineering of vesicles has already been done with Nm (Koeberling et al., 2009). Together, this body of work represents steps towards a process by which protective mechanisms elucidated in next generation murine

models of GC infection are deconstructed to inform and direct the design of the next generation of *Neisseria gonorrhoeae* nOMV vaccines.

Bibliography

(2007). Update to CDC's Sexually Transmitted Diseases Treatment Guidelines, 2006: Fluoroquinolones No Longer Recommended for Treatment of Gonococcal Infections. U.S.D.o.H.a.H. Services, editor Center for Surveillance, Epidemiology, and Laboratory Services, Atlanta. 332-336.

(2014). STI diagnoses and rates in England by gender, 2004-2013 H.S.T.I. Department, editor Public Health England, London.

(2016). New Warning Signs that Gonorrhea Treatment May be Losing Effectiveness. Center for Disease Control and Prevention.

(2017). 2016 Sexually Transmitted Diseases Surveillance. S.a.D.M.B.-D.o.S. Prevention, editor U.S. Department of Health and Human Services, Atlanta.

Acevedo, R., S. Fernandez, C. Zayas, A. Acosta, M.E. Sarmiento, V.A. Ferro, E. Rosenqvist, C. Campa, D. Cardoso, L. Garcia, and J.L. Perez. (2014). Bacterial outer membrane vesicles and vaccine applications. *Frontiers in Immunology,* 5:121.

Allen, V.G., L. Mitterni, C. Seah, A. Rebbapragada, I.E. Martin, C. Lee, H. Siebert, L. Towns, R.G. Melano, and D.E. Low. (2013). Neisseria gonorrhoeae treatment failure and susceptibility to cefixime in Toronto, Canada. *The Journal of the American Medical Association,* 309:163-170.

Amies, C.R. (1967). Development of resistance of gonococci to penicillin: an eight-year study. *Canadian Medical Association Journal,* 96:33-35.

Andrade, W.A., S. Agarwal, S. Mo, S.A. Shaffer, J.P. Dillard, T. Schmidt, V. Hornung, K.A. Fitzgerald, E.A. Kurt-Jones, and D.T. Golenbock. (2016). Type I Interferon Induction by Neisseria gonorrhoeae: Dual Requirement of Cyclic GMP-AMP Synthase and Toll-like Receptor 4. *Cell Reports,* 15:2438-2448.

Arigita, C., W. Jiskoot, J. Westdijk, C. van Ingen, W.E. Hennink, D.J. Crommelin, and G.F. Kersten. (2004). Stability of mono- and trivalent meningococcal outer membrane vesicle vaccines. *Vaccine,* 22:629-642.

Arko, R.J. (1989). Animal models for pathogenic Neisseria species. *Clinical microbiology reviews,* 2 Suppl:S56-59.

Ashford, W.A., R.G. Golash, and V.G. Hemming. (1976). Penicillinase-producing Neisseria gonorrhoeae. *Lancet,* 2:657-658.

Bala, M., M. Kakran, V. Singh, S. Sood, V. Ramesh, and W.H.O.G.S.N. Members of. (2013). Monitoring antimicrobial resistance in Neisseria gonorrhoeae in selected countries of the WHO South-East Asia Region between 2009 and 2012: a retrospective analysis. *Sexually transmitted infections,* 89 Suppl 4:iv28-35.

Barlow, D., and I. Phillips. (1978). Gonorrhoea in women. Diagnostic, clinical, and laboratory aspects. *Lancet,* 1:761-764.

Becker, E.L. (1987). Rous-Whipple award lecture. The formylpeptide receptor of the neutrophil. A search and conserve operation. *The American journal of pathology,* 129:15-24.

Belkaid, Y., and T. Hand. (2014). Role of the Microbiota in Immunity and inflammation. *Cell,* 157:121-141.

Belyakov, I.M., and J.D. Ahlers. (2009). What role does the route of immunization play in the generation of protective immunity against mucosal pathogens? *The Journal of immunology,* 183:6883-6892.

Besredka, A. (1919). De la vaccination contre les états typhoides par la voie buccale. *Annales De l'Institut Pasteur,* 882-903.

Bignold, L.P., S.D. Rogers, T.M. Siaw, and J. Bahnisch. (1991). Inhibition of chemotaxis of neutrophil leukocytes to interleukin-8 by endotoxins of various bacteria. *Infection and immunity,* 59:4255-4258.

Bingham, J.S. (2014). Historical Aspects of Sexually Transmitted Infections. In Sexually Transmitted Infections. B. Kumar, and S. Gupta, editors. Elsevier Health Sciences,

Blake, M.S., and E.C. Gotschlich. (1982). Purification and partial characterization of the major outer membrane protein of Neisseria gonorrhoeae. *Infection and immunity,* 36:277-283.

Blake, M.S., L.M. Wetzler, E.C. Gotschlich, and P.A. Rice. (1989). Developing a gonococcal protein I vaccine. *Advances in experimental medicine and biology,* 251:315-327.

Borrego, F. (2013). The CD300 molecules: an emerging family of regulators of the immune system. *Blood,* 121:1951-1960.

Bos, M.P., F. Grunert, and R.J. Belland. (1997). Differential recognition of members of the carcinoembryonic antigen family by Opa variants of Neisseria gonorrhoeae. *Infection and immunity,* 65:2353-2361.

Bos, M.P., V. Robert, and J. Tommassen. (2007). Biogenesis of the gram-negative bacterial outer membrane. *Annual review of microbiology,* 61:191-214.

Boslego, J.W., E.C. Tramont, R.C. Chung, D.G. McChesney, J. Ciak, J.C. Sadoff, M.V. Piziak, J.D. Brown, C.C. Brinton, Jr., S.W. Wood, and et al. (1991). Efficacy trial of a parenteral gonococcal pilus vaccine in men. *Vaccine,* 9:154-162.

Boulay, F., M. Tardif, L. Brouchon, and P. Vignais. (1990). The human N-formylpeptide receptor. Characterization of two cDNA isolates and evidence for a new subfamily of G-protein-coupled receptors. *Biochemistry,* 29:11123-11133.

Brandtzaeg, P. (2007). Induction of secretory immunity and memory at mucosal surfaces. *Vaccine,* 25:5467-5484.

Brandtzaeg, P. (2013). Secretory IgA: Designed for Anti-Microbial Defense. *Frontiers in Immunology,* 4:222.

Braude, A.I., L.B. Corbeil, S. Levine, J. Ito, and J.A. McCutchan. (1978). Possible influence of cyclic menstrual changes on resistance to the gonococcus. In Immunobiology of Neisseria gonorrhoeae. G.F. Brooks, E.C. Gotschlich, K.K. Holmes, W.D. Sawer, and F.E. Young, editors. American Society for Microbiology, Washington, D.C.

Bryan, C.P. (1930). Ancient Egyptian Medicine: The Papyrus Ebers. Ares Publishers, Chicago.

Burns, A.R., R.A. Bowden, Y. Abe, D.C. Walker, S.I. Simon, M.L. Entman, and C.W. Smith. (1999). P-selectin mediates neutrophil adhesion to endothelial cell borders. *Journal of leukocyte biology,* 65:299-306.

Caligioni, C.S. (2009). Assessing reproductive status/stages in mice. *Current protocols in neuroscience,* Appendix 4:Appendix 4I.

Camara, J., J. Serra, J. Ayats, T. Bastida, D. Carnicer-Pont, A. Andreu, and C. Ardanuy. (2012). Molecular characterization of two high-level ceftriaxone-resistant Neisseria gonorrhoeae isolates detected in Catalonia, Spain. *The Journal of antimicrobial chemotherapy,* 67:1858-1860.

Carmeli, Y.H., S.; Kahlmeter, G.; Kluytmans, J.; Mendelson, M.; Pulcini, C.; Singh, N.; Theuretzbacher, U. (2017). GLOBAL PRIORITY LIST OF ANTIBIOTIC-RESISTANT BACTERIA TO GUIDE RESEARCH, DISCOVERY, AND DEVELOPMENT OF NEW ANTIBIOTICS. N.M. E. Tacconelli, editor World Health Organization.

CDC. (2012). Antibiotic Resistance Threats in the United States, 2013. U.S.D.o.H.a.H. Services, editor.

Chan, C.H., and C.P. Stanners. (2004). Novel mouse model for carcinoembryonic antigen-based therapy. *Molecular therapy,* 9:775-785.

Chatterjee, S.N., and J. Das. (1967). Electron microscopic observations on the excretion of cell-wall material by Vibrio cholerae. *Journal of general microbiology,* 49:1-11.

Chavakis, T., T. Keiper, R. Matz-Westphal, K. Hersemeyer, U.J. Sachs, P.P. Nawroth, K.T. Preissner, and S. Santoso. (2004). The junctional adhesion molecule-C promotes neutrophil transendothelial migration in vitro and in vivo. *The Journal of biological chemistry,* 279:55602-55608.

Chisholm, S.A., J. Dave, and C.A. Ison. (2010). High-level azithromycin resistance occurs in Neisseria gonorrhoeae as a result of a single point mutation in the 23S rRNA genes. *Antimicrobial agents and chemotherapy,* 54:3812-3816.

Chow, J.C., D.W. Young, D.T. Golenbock, W.J. Christ, and F. Gusovsky. (1999). Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *The Journal of biological chemistry,* 274:10689-10692.

Claassen, I., J. Meylis, P. van der Ley, C. Peeters, H. Brons, J. Robert, D. Borsboom, A. van der Ark, I. van Straaten, P. Roholl, B. Kuipers, and J. Poolman. (1996). Production, characterization and control of a Neisseria meningitidis hexavalent class 1 outer membrane protein containing vesicle vaccine. *Vaccine,* 14:1001-1008.

Clark, V.L., L.A. Campbell, D.A. Palermo, T.M. Evans, and K.W. Klimpel. (1987). Induction and repression of outer membrane proteins by anaerobic growth of Neisseria gonorrhoeae. *Infection and immunity,* 55:1359-1364.

Cook, D.N., D.M. Prosser, R. Forster, J. Zhang, N.A. Kuklin, S.J. Abbondanzo, X.D. Niu, S.C. Chen, D.J. Manfra, M.T. Wiekowski, L.M. Sullivan, S.R. Smith, H.B. Greenberg, S.K. Narula, M. Lipp, and S.A. Lira. (2000). CCR6 mediates dendritic cell localization, lymphocyte homeostasis, and immune responses in mucosal tissue. *Immunity,* 12:495-503.

Coordinators, N.R. (2013). Database resources of the National Center for Biotechnology Information. *Nucleic acids research,* 41:D8-D20.

Corbeil, L.B., A. Chatterjee, L. Foresman, and J.A. Westfall. (1985). Ultrastructure of cyclic changes in the murine uterus, cervix, and vagina. *Tissue & Cell,* 17:53-68.

Crossin, K.L., and L.A. Krushel. (2000). Cellular signaling by neural cell adhesion molecules of the immunoglobulin superfamily. *Developmental dynamics,* 218:260-279.

Dai, M., P. Wang, A.D. Boyd, G. Kostov, B. Athey, E.G. Jones, W.E. Bunney, R.M. Myers, T.P. Speed, H. Akil, S.J. Watson, and F. Meng. (2005). Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data. *Nucleic acids research,* 33:e175.

Davies, A. (1922). AN INVESTIGATION INTO THE SEROLOGICAL PROPERTIES OF DYSENTERY STOOLS. *The Lancet,* 200:1009-1012.

De Temmerman, G., G.M. Villeirs, and K.L. Verstraete. (2003). Ruptured tuboovarian abscess causing peritonitis in a postmenopausal woman. A difficult diagnosis on imaging. *Journal belge de radiologie*
Belgisch tijdschrift voor radiologie, 86:72-73.

de Vries, H.J., A. Zingoni, J.A. White, J.D. Ross, and A. Kreuter. (2014). 2013 European Guideline on the management of proctitis, proctocolitis and enteritis caused by sexually transmissible pathogens. *International journal of STD & AIDS,* 25:465-474.

Deguchi, T., M. Yasuda, and S. Ito. (2012). Management of pharyngeal gonorrhea is crucial to prevent the emergence and spread of antibiotic-resistant Neisseria gonorrhoeae. *Antimicrobial agents and chemotherapy,* 56:4039-4040; author re[ply 4041-4032.

Deguchi, T., M. Yasuda, S. Yokoi, K. Ishida, M. Ito, S. Ishihara, K. Minamidate, Y. Harada, K. Tei, K. Kojima, M. Tamaki, and S. Maeda. (2003). Treatment of uncomplicated gonococcal urethritis by double-dosing of 200 mg cefixime at a 6-h interval. *Journal of infection and chemotherapy,* 9:35-39.

Dillon, J.A., M.A. Trecker, S.D. Thakur, A. Gonococcal Antimicrobial Surveillance Program Network in Latin, and Caribbean. (2013). Two decades of the gonococcal antimicrobial surveillance program in South America and the Caribbean: challenges and opportunities. *Sexually transmitted infections,* 89 Suppl 4:iv36-41.

Ding, Y., J. Li, Q. Wu, P. Yang, B. Luo, S. Xie, K.M. Druey, A.J. Zajac, H.C. Hsu, and J.D. Mountz. (2013). IL-17RA is essential for optimal localization of follicular Th cells in the germinal center light zone to promote autoantibody-producing B cells. *The Journal of immunology,* 191:1614-1624.

Dobson-Belaire, W.N., A. Rebbapragada, R.J. Malott, F.Y. Yue, C. Kovacs, R. Kaul, M.A. Ostrowski, and S.D. Gray-Owen. (2010). Neisseria gonorrhoeae effectively

blocks HIV-1 replication by eliciting a potent TLR9-dependent interferon-alpha response from plasmacytoid dendritic cells. *Cellular microbiology,* 12:1703-1717.

Dockery, P., J. Khalid, S.A. Sarani, H.E. Bulut, M.A. Warren, T.C. Li, and I.D. Cooke. (1998). Changes in basement membrane thickness in the human endometrium during the luteal phase of the menstrual cycle. *Human reproduction update,* 4:486-495.

Donadoni, C., C. Bisighini, L. Scotti, L. Diomede, M. Ngyen, J. Nouhin, L. DeSantis, A. Zambon, D. Ferrari, G. Gallotta, G. Corrao, G. Pancino, and L. Lopalco. (2010). Setting of Methods for Analysis of Mucosal Antibodies in Seminal and Vaginal Fluids of HIV Seropositive Subjects from Cambodian and Italian Cohorts. *PLoS ONE,* 5:e9920.

Dorward, D.W., and R.C. Judd. (1988). The isolation and partial characterization of naturally-evolved outer membrane blebs of Neisseria gonorrhoeae. In Gonococci and Meningococci: Epidemiology, Genetics, Immunochemistry and Pathogenesis. J.T. Poolman, editor Springer Netherlands, Dordrecht. 349-356.

Dunlop, E.M. (1949). Gonorrhoea and the sulphonamides. *The British journal of venereal diseases,* 25:81-83.

Edwards, J.L., E.J. Brown, K.A. Ault, and M.A. Apicella. (2001). The role of complement receptor 3 (CR3) in Neisseria gonorrhoeae infection of human cervical epithelia. *Cellular microbiology,* 3:611-622.

Edwards, J.L., and E.K. Butler. (2011). The Pathobiology of Neisseria gonorrhoeae Lower Female Genital Tract Infection. *Frontiers in microbiology,* 2:102.

Eisenstein, T.K., L.M. Killar, and B.M. Sultzer. (1984). Immunity to Infection with Salmonella typhimurium: Mouse-Strain Differences in Vaccine- and Serum-Mediated Protection. *The Journal of Infectious Diseases,* 150:425-435.

Ellis, T.N., and M.J. Kuehn. (2010). Virulence and immunomodulatory roles of bacterial outer membrane vesicles. *Microbiology and molecular biology reviews,* 74:81-94.

Evans, B.A. (1977). Ultrastructural study of cervical gonorrhea. *The Journal of Infectious Diseases,* 136:248-255.

Fahrbach, K.M., O. Malykhina, D.J. Stieh, and T.J. Hope. (2013). Differential Binding of IgG and IgA to Mucus of the Female Reproductive Tract. *PLoS ONE,* 8:e76176.

Faruki, H., R.N. Kohmescher, W.P. McKinney, and P.F. Sparling. (1985). A community-based outbreak of infection with penicillin-resistant Neisseria gonorrhoeae not

producing penicillinase (chromosomally mediated resistance). *The New England journal of medicine,* 313:607-611.

Faruki, H., and P.F. Sparling. (1986). Genetics of resistance in a non-beta-lactamase-producing gonococcus with relatively high-level penicillin resistance. *Antimicrobial agents and chemotherapy,* 30:856-860.

Fenton, K.A., and C.M. Lowndes. (2004). Recent trends in the epidemiology of sexually transmitted infections in the European Union. *Sexually transmitted infections,* 80:255-263.

Figueroa, J.E., and P. Densen. (1991). Infectious diseases associated with complement deficiencies. *Clinical microbiology reviews,* 4:359-395.

Finne, J., M. Leinonen, and P.H. Makela. (1983). Antigenic similarities between brain components and bacteria causing meningitis. Implications for vaccine development and pathogenesis. *Lancet,* 2:355-357.

Floudas, A., S.P. Saunders, T. Moran, C. Schwartz, E. Hams, D.C. Fitzgerald, J.A. Johnston, G.S. Ogg, A.N. McKenzie, P.T. Walsh, and P.G. Fallon. (2017). IL-17 Receptor A Maintains and Protects the Skin Barrier To Prevent Allergic Skin Inflammation. *The Journal of immunology,* 199:707-717.

Franks, A.G. (1946). Successful combined treatment of penicillin-resistant gonorrhea. *The American journal of the medical sciences,* 211:553-555.

Fudyk, T.C., I.W. Maclean, J.N. Simonsen, E.N. Njagi, J. Kimani, R.C. Brunham, and F.A. Plummer. (1999). Genetic diversity and mosaicism at the por locus of Neisseria gonorrhoeae. *Journal of bacteriology,* 181:5591-5599.

Fukuyama, Y., Y. Ikeda, J. Ohori, G. Sugita, K. Aso, K. Fujihashi, D.E. Briles, J.R. McGhee, and K. Fujihashi. (2015). A Molecular Mucosal Adjuvant To Enhance Immunity Against Pneumococcal Infection In The Elderly. *Immune Network,* 15:9-15.

Gautier, L., L. Cope, B.M. Bolstad, and R.A. Irizarry. (2004). affy--analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics,* 20:307-315.

Gawish, R., R. Martins, B. Bohm, T. Wimberger, O. Sharif, K. Lakovits, M. Schmidt, and S. Knapp. (2015). Triggering receptor expressed on myeloid cells-2 fine-tunes inflammatory responses in murine Gram-negative sepsis. *Federation of American Societies for Experimental Biology journal,* 29:1247-1257.

Genco, C.A., and L.M. Wetzler. (2010). Neisseria: Molecular Mechanisms of Pathogenesis. Caister Academic Press,

Gentleman, R.C., V.J. Carey, D.M. Bates, B. Bolstad, M. Dettling, S. Dudoit, B. Ellis, L. Gautier, Y. Ge, J. Gentry, K. Hornik, T. Hothorn, W. Huber, S. Iacus, R. Irizarry, F. Leisch, C. Li, M. Maechler, A.J. Rossini, G. Sawitzki, C. Smith, G. Smyth, L. Tierney, J.Y. Yang, and J. Zhang. (2004). Bioconductor: open software development for computational biology and bioinformatics. *Genome biology,* 5:R80.

Ghanem, K. (2015). Clinical manifestations and diagnosis of Neisseria gonorrhoeae infection in adults and adolescents. Up To Date. Wolters Klewer.

Gondek, D.C., A.J. Olive, G. Stary, and M.N. Starnbach. (2012). CD4(+) T cells are necessary and sufficient to confer protection against C. trachomatis infection in the murine upper genital tract. *The Journal of immunology,* 189:2441-2449.

Granoff, D.M. (2009). Relative importance of complement-mediated bactericidal and opsonic activity for protection against meningococcal disease. *Vaccine,* 27 Suppl 2:B117-125.

Granoff, D.M. (2010). Review of Meningococcal Group B Vaccines. *Clinical infectious diseases,* 50:S54.

Gray-Owen, S.D., and A.B. Schryvers. (1993). The interaction of primate transferrins with receptors on bacteria pathogenic to humans. *Microbial pathogenesis,* 14:389-398.

Gu, A., Z. Zhang, N. Zhang, W. Tsark, and J.E. Shively. (2010). Generation of human CEACAM1 transgenic mice and binding of Neisseria Opa protein to their neutrophils. *PLoS One,* 5:e10067.

Guglani, L., and S.A. Khader. (2010). Th17 cytokines in mucosal immunity and inflammation. *Current opinion in HIV and AIDS,* 5:120-127.

Gulati, S., P.A. Rice, M.S. Blake, S.K. Sarafian, S.A. Morse, M.J. Quentin-Millet, F. Arminjon, M. Achtman, P.K. Kohl, C. Marchal, G. Morelli, A. Seiler, and B. Thiesen. (1991). Antibody responses in six volunteers immunized with a gonococcal Protein I vaccine. *In Neisseriae 1990 - Proceedings of the Seventh International Pathogenic Neisseria Conference, Berlin, Federal Republic of Germany, September 9-14, 1990,* 229-234.

Haddad, S.N., and C.R. Wira. (2014). Estradiol regulation of constitutive and keratinocyte growth factor-induced CCL20 and CXCL1 secretion by mouse uterine epithelial cells. *American journal of reproductive immunology,* 72:34-44.

Haggerty, C.L., S.L. Gottlieb, B.D. Taylor, N. Low, F. Xu, and R.B. Ness. (2010). Risk of sequelae after Chlamydia trachomatis genital infection in women. *The Journal of infectious diseases,* 201 Suppl 2:S134-155.

Hammarstrom, S. (1999). The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues. *Seminars in cancer biology,* 9:67-81.

Handsfield, H.H., T.O. Lipman, J.P. Harnisch, E. Tronca, and K.K. Holmes. (1974). Asymptomatic gonorrhea in men. Diagnosis, natural course, prevalence and significance. *The New England journal of medicine,* 290:117-123.

Harrison, W.O., R.R. Hooper, P.J. Wiesner, A.F. Campbell, W.W. Karney, G.H. Reynolds, O.G. Jones, and K.K. Holmes. (1979). A trial of minocycline given after exposure to prevent gonorrhea. *The New England journal of medicine,* 300:1074-1078.

Hasegawa, H., T. Ichinohe, A. Ainai, S. Tamura, and T. Kurata. (2009). Development of mucosal adjuvants for intranasal vaccine for H5N1 influenza viruses. *Therapeutics and clinical risk management,* 5:125-132.

Hauck, C.R., T.F. Meyer, F. Lang, and E. Gulbins. (1998). CD66-mediated phagocytosis of Opa52 Neisseria gonorrhoeae requires a Src-like tyrosine kinase- and Rac1-dependent signalling pathway. *The EMBO journal,* 17:443-454.

Hawkins, S.M., and M.M. Matzuk. (2008). Menstrual Cycle: Basic Biology. *Annals of the New York Academy of Sciences,* 1135:10-18.

Hebb, J.K., C.R. Cohen, S.G. Astete, E.A. Bukusi, and P.A. Totten. (2004). Detection of novel organisms associated with salpingitis, by use of 16S rDNA polymerase chain reaction. *The Journal of Infectious Diseases,* 190:2109-2120.

Heckels, J.E., J.N. Fletcher, and M. Virji. (1989). The potential protective effect of immunization with outer-membrane protein I from Neisseria gonorrhoeae. *Journal of general microbiology,* 135:2269-2276.

Hess, D., A. Wu, D. Golparian, S. Esmaili, W. Pandori, E. Sena, J.D. Klausner, P. Barry, M. Unemo, and M. Pandori. (2012). Genome sequencing of a Neisseria gonorrhoeae isolate of a successful international clone with decreased susceptibility and resistance to extended-spectrum cephalosporins. *Antimicrobial agents and chemotherapy,* 56:5633-5641.

Hieshima, K., H. Ohtani, M. Shibano, D. Izawa, T. Nakayama, Y. Kawasaki, F. Shiba, M. Shiota, F. Katou, T. Saito, and O. Yoshie. (2003). CCL28 has dual roles in

mucosal immunity as a chemokine with broad-spectrum antimicrobial activity. *The Journal of Immunology,* 170:1452-1461.

Holmes, K.K., R.E. Berger, and E.R. Alexander. (1979). Acute epididymitis: etiology and therapy. *Archives of andrology,* 3:309-316.

Hoover, D.M., C. Boulegue, D. Yang, J.J. Oppenheim, K. Tucker, W. Lu, and J. Lubkowski. (2002). The structure of human macrophage inflammatory protein-3alpha /CCL20. Linking antimicrobial and CC chemokine receptor-6-binding activities with human beta-defensins. *The Journal of biological chemistry,* 277:37647-37654.

Administering Vaccines: Dose, Route, Site, and Needle size. (2018). www.immunize.org.

Irizarry, R.A., B. Hobbs, F. Collin, Y.D. Beazer-Barclay, K.J. Antonellis, U. Scherf, and T.P. Speed. (2003). Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics,* 4:249-264.

Islam, E., V. Anipindi, I. Francis, Y. Shaik-Dasthagirisaheb, S. Xu, N. Leung, A. Sintsova, M. Amin, C. Kaushic, L. Wetzler, and S. Gray-Owen. (2018). Specific binding to differentially-expressed human CEACAMs determines the outcome of gonococcal infections along the female reproductive tract.

Islam, E., Y. Shaik-Dasthagirisaheb, C. Kaushic, L. Wetzler, and S.D. Gray-Owen. (2016). The Reproductive Cycle is a Pathogenic Determinant during Gonococcal Pelvic Inflammatory Disease in Mice. *Mucosal Immunology,* 9:1051-1064.

Ison, C.A., J. Hussey, K.N. Sankar, J. Evans, and S. Alexander. (2011). Gonorrhoea treatment failures to cefixime and azithromycin in England, 2010. *Euro surveillance : bulletin Européen sur les maladies transmissibles,* 16:

Jaffe, H.W., J.W. Biddle, C. Thornsberry, R.E. Johnson, R.E. Kaufman, G.H. Reynolds, and P.J. Wiesner. (1976). National gonorrhea therapy monitoring study: in vitro antibiotic susceptibility and its correlation with treatment results. *The New England journal of medicine,* 294:5-9.

Jerse, A.E. (1999). Experimental gonococcal genital tract infection and opacity protein expression in estradiol-treated mice. *Infection and immunity,* 67:5699-5708.

Jerse, A.E., and C.D. Deal. (2013). Vaccine research for gonococcal infections: where are we? *Sexually transmitted infections,* 89 Suppl 4:iv63-68.

Jerse, A.E., H. Wu, M. Packiam, R.A. Vonck, A.A. Begum, and L.E. Garvin. (2011). Estradiol-Treated Female Mice as Surrogate Hosts for Neisseria gonorrhoeae Genital Tract Infections. *Frontiers in microbiology,* 2:107.

Johansson, M., and N.Y. Lycke. (2003). Immunology of the human genital tract. *Current opinion in infectious diseases,* 16:43-49.

John, J., and W.H. Donald. (1978). Asymptomatic urethral gonorrhoea in men. *The British journal of venereal diseases,* 54:322-323.

Johnson, A.P., D. Taylor-Robinson, and Z.A. McGee. (1977). Species specificity of attachment and damage to oviduct mucosa by Neisseria gonorrhoeae. *Infection and immunity,* 18:833-839.

Joiner, K.A., R. Scales, K.A. Warren, M.M. Frank, and P.A. Rice. (1985). Mechanism of action of blocking immunoglobulin G for Neisseria gonorrhoeae. *The Journal of clinical investigation,* 76:1765-1772.

Kampmeier, R.H. (1983). Introduction of sulfonamide therapy for gonorrhea. *Sexually transmitted diseases,* 10:81-84.

Kaparakis, M., L. Turnbull, L. Carneiro, S. Firth, H.A. Coleman, H.C. Parkington, L. Le Bourhis, A. Karrar, J. Viala, J. Mak, M.L. Hutton, J.K. Davies, P.J. Crack, P.J. Hertzog, D.J. Philpott, S.E. Girardin, C.B. Whitchurch, and R.L. Ferrero. (2010). Bacterial membrane vesicles deliver peptidoglycan to NOD1 in epithelial cells. *Cellular microbiology,* 12:372-385.

Kaproth-Joslin, K., and V. Dogra. (2013). Imaging of female infertility: a pictorial guide to the hysterosalpingography, ultrasonography, and magnetic resonance imaging findings of the congenital and acquired causes of female infertility. *Radiologic clinics of North America,* 51:967-981.

Kasper, D.L., A.S. Fauci, S.L. Hauser, D.L. Longo, J.L. Jameson, and J. Loscalzo. (2016). Sexually Transmitted and Reproductive Tract Infections. In Harrison's Manual of Medicine, 19e. McGraw-Hill Education, New York, NY.

Katz, A.R., A.Y. Komeya, O.O. Soge, M.I. Kiaha, M.V. Lee, G.M. Wasserman, E.V. Maningas, A.C. Whelen, R.D. Kirkcaldy, S.J. Shapiro, G.A. Bolan, and K.K. Holmes. (2012). Neisseria gonorrhoeae with high-level resistance to azithromycin: case report of the first isolate identified in the United States. *Clinical infectious diseases,* 54:841-843.

Keiser, P.B., B.T. Gibbs, T.S. Coster, E.E. Moran, M.B. Stoddard, J.E. Labrie, 3rd, D.H. Schmiel, V. Pinto, P. Chen, and W.D. Zollinger. (2010). A phase 1 study of a group B meningococcal native outer membrane vesicle vaccine made from a strain with deleted lpxL2 and synX and stable expression of opcA. *Vaccine,* 28:6970-6976.

Kent, C.K., J.K. Chaw, W. Wong, S. Liska, S. Gibson, G. Hubbard, and J.D. Klausner. (2005). Prevalence of rectal, urethral, and pharyngeal chlamydia and gonorrhea detected in 2 clinical settings among men who have sex with men: San Francisco, California, 2003. *Clinical infectious diseases,* 41:67-74.

Kirkcaldy, R.D., S. Kidd, H.S. Weinstock, J.R. Papp, and G.A. Bolan. (2013). Trends in antimicrobial resistance in Neisseria gonorrhoeae in the USA: the Gonococcal Isolate Surveillance Project (GISP), January 2006-June 2012. *Sexually transmitted diseases,* 89 Suppl 4:iv5-10.

Kirkcaldy, R.D., C. Rio, G. Hall, E.W. Hook, W. Whittington, H.S. Weinstock, and E. Murray. (2011). Cephalosporin Susceptibility Among Neisseria gonorrhoeae Isolates — United States, 2000–2010. U.S.D.o.H.a.H. Services, editor Center for Surveillance, Epidemiology, and Laboratory Services, Atlanta. 873-877.

Klein, E.J., L.S. Fisher, A.W. Chow, and L.B. Guze. (1977). Anorectal gonococcal infection. *Annals of internal medicine,* 86:340-346.

Klimentova, J., and J. Stulik. (2015). Methods of isolation and purification of outer membrane vesicles from gram-negative bacteria. *Microbiological research,* 170:1-9.

Klouman, E., E.J. Masenga, N.E. Sam, and K.I. Klepp. (2000). Asymptomatic gonorrhoea and chlamydial infection in a population-based and work-site based sample of men in Kilimanjaro, Tanzania. *International journal of STD & AIDS,* 11:666-674.

Koeberling, O., S. Giuntini, A. Seubert, and D.M. Granoff. (2009). Meningococcal Outer Membrane Vesicle Vaccines Derived from Mutant Strains Engineered To Express Factor H Binding Proteins from Antigenic Variant Groups 1 and 2. *Clinical and Vaccine Immunology,* 16:156-162.

Koeppen, K., T.H. Hampton, M. Jarek, M. Scharfe, S.A. Gerber, D.W. Mielcarz, E.G. Demers, E.L. Dolben, J.H. Hammond, D.A. Hogan, and B.A. Stanton. (2016). A Novel Mechanism of Host-Pathogen Interaction through sRNA in Bacterial Outer Membrane Vesicles. *PLoS Pathogens,* 12:e1005672.

Komaroff, A.L., M.D. Aronson, T.M. Pass, and C.T. Ervin. (1980). Prevalence of pharyngeal gonorrhea in general medical patients with sore throats. *Sexually transmitted diseases,* 7:116-119.

Kool, M., K. Fierens, and B.N. Lambrecht. (2012). Alum adjuvant: some of the tricks of the oldest adjuvant. *Journal of medical microbiology,* 61:927-934.

Korsholm, K.S., R.V. Petersen, E.M. Agger, and P. Andersen. (2010). T-helper 1 and T-helper 2 adjuvants induce distinct differences in the magnitude, quality and kinetics of the early inflammatory response at the site of injection. *Immunology,* 129:75-86.

Kraus, S.J. (1979). Incidence and therapy of gonococcal pharyngitis. *Sexually transmitted diseases,* 6:143-147.

Kraus, S.J., W.J. Brown, and R.J. Arko. (1975). Acquired and natural immunity to gonococcal infection in chimpanzees. *The Journal of clinical investigation,* 55:1349-1356.

Kretschmer, D., A.K. Gleske, M. Rautenberg, R. Wang, M. Koberle, E. Bohn, T. Schoneberg, M.J. Rabiet, F. Boulay, S.J. Klebanoff, K.A. van Kessel, J.A. van Strijp, M. Otto, and A. Peschel. (2010). Human formyl peptide receptor 2 senses highly pathogenic Staphylococcus aureus. *Cell host and microbe,* 7:463-473.

Kuehn, M.J., and N.C. Kesty. (2005). Bacterial outer membrane vesicles and the host-pathogen interaction. *Genes & development,* 19:2645-2655.

Kuespert, K., S. Pils, and C.R. Hauck. (2006). CEACAMs: their role in physiology and pathophysiology. *Current opinion in cell biology,* 18:565-571.

Kulp, A., and M.J. Kuehn. (2010). Biological functions and biogenesis of secreted bacterial outer membrane vesicles. *Annual review of microbiology,* 64:163-184.

Kutchel, P.W., and G.B. Ralston. (1988). Theory and problems Biochemistry.

Lahra, M.M., Y.R. Lo, and D.M. Whiley. (2013). Gonococcal antimicrobial resistance in the Western Pacific Region. *Sexually transmitted infections,* 89 Suppl 4:iv19-23.

Lappann, M., A. Otto, D. Becher, and U. Vogel. (2013). Comparative proteome analysis of spontaneous outer membrane vesicles and purified outer membranes of Neisseria meningitidis. *Journal of bacteriology,* 195:4425-4435.

Lazarus, N.H., E.J. Kunkel, B. Johnston, E. Wilson, K.R. Youngman, and E.C. Butcher. (2003). A common mucosal chemokine (mucosae-associated epithelial chemokine/CCL28) selectively attracts IgA plasmablasts. *The Journal of Immunology,* 170:3799-3805.

Lee, B.C., and A.B. Schryvers. (1988). Specificity of the lactoferrin and transferrin receptors in Neisseria gonorrhoeae. *Molecular Microbiology,* 2:827-829.

Lee, H.S., I.C. Boulton, K. Reddin, H. Wong, D. Halliwell, O. Mandelboim, A.R. Gorringe, and S.D. Gray-Owen. (2007). Neisserial outer membrane vesicles bind

the coinhibitory receptor carcinoembryonic antigen-related cellular adhesion molecule 1 and suppress CD4+ T lymphocyte function. *Infection and immunity,* 75:4449-4455.

Lee, J., G. Cacalano, T. Camerato, K. Toy, M.W. Moore, and W.I. Wood. (1995). Chemokine binding and activities mediated by the mouse IL-8 receptor. *The Journal of Immunology,* 155:2158-2164.

Lee, J., O.Y. Kim, and Y.S. Gho. (2016). Proteomic profiling of Gram-negative bacterial outer membrane vesicles: Current perspectives. *Proteomics. Clinical applications,* 10:897-909.

Leroux-Roels, G. (2010). Unmet needs in modern vaccinology: adjuvants to improve the immune response. *Vaccine,* 28 Suppl 3:C25-36.

Lewis, D.A. (2010). The Gonococcus fights back: is this time a knock out? *Sexually transmitted infections,* 86:415-421.

Lewis, D.A., C. Sriruttan, E.E. Muller, D. Golparian, L. Gumede, D. Fick, J. de Wet, V. Maseko, J. Coetzee, and M. Unemo. (2013). Phenotypic and genetic characterization of the first two cases of extended-spectrum-cephalosporin-resistant Neisseria gonorrhoeae infection in South Africa and association with cefixime treatment failure. *The Journal of antimicrobial chemotherapy,* 68:1267-1270.

Lewis, L.A., S. Gulati, E. Burrowes, B. Zheng, S. Ram, and P.A. Rice. (2015). alpha-2,3-sialyltransferase expression level impacts the kinetics of lipooligosaccharide sialylation, complement resistance, and the ability of Neisseria gonorrhoeae to colonize the murine genital tract. *MBio,* 6:

Li, S.Y. (2012). Global transmission of multiple-drug resistant Neisseria gonorrhoeae strains refractive to cephalosporin treatment. *Journal of the Formosan Medical Association,* 111:463-464.

Li, Z., S. Palaniyandi, R. Zeng, W. Tuo, D.C. Roopenian, and X. Zhu. (2011). Transfer of IgG in the female genital tract by MHC class I-related neonatal Fc receptor (FcRn) confers protective immunity to vaginal infection. *Proceedings of the National Academy of Sciences of the United States of America,* 108:4388-4393.

Lindmark, B., P.K. Rompikuntal, K. Vaitkevicius, T. Song, Y. Mizunoe, B.E. Uhlin, P. Guerry, and S.N. Wai. (2009). Outer membrane vesicle-mediated release of cytolethal distending toxin (CDT) from Campylobacter jejuni. *BioMed Central microbiology,* 9:220.

Liu, X., L.M. Wetzler, and P. Massari. (2008). The PorB porin from commensal Neisseria lactamica induces Th1 and Th2 immune responses to ovalbumin in mice and is a potential immune adjuvant. *Vaccine,* 26:786-796.

Liu, Y., J. Perez, L.A. Hammer, H.C. Gallagher, M. De Jesus, N.K. Egilmez, and M.W. Russell. (2018). Intravaginal Administration of Interleukin 12 during Genital Gonococcal Infection in Mice Induces Immunity to Heterologous Strains of Neisseria gonorrhoeae. *mSphere,* 3:

Lo, J.Y., K.M. Ho, A.O. Leung, F.S. Tiu, G.K. Tsang, A.C. Lo, and J.W. Tapsall. (2008). Ceftibuten resistance and treatment failure of Neisseria gonorrhoeae infection. *Antimicrobial agents and chemotherapy,* 52:3564-3567.

Lomakina, E.B., and R.E. Waugh. (2009). Adhesion between human neutrophils and immobilized endothelial ligand vascular cell adhesion molecule 1: divalent ion effects. *Biophysical journal,* 96:276-284.

Lombard, R., E. Doz, F. Carreras, M. Epardaud, Y. Le Vern, D. Buzoni-Gatel, and N. Winter. (2016). IL-17RA in Non-Hematopoietic Cells Controls CXCL-1 and 5 Critical to Recruit Neutrophils to the Lung of Mycobacteria-Infected Mice during the Adaptive Immune Response. *PLoS One,* 11:e0149455.

Lytton, E.J., and M.S. Blake. (1986). Isolation and partial characterization of the reduction-modifiable protein of Neisseria gonorrhoeae. *The Journal of experimental medicine.,* 164:1749-1759.

MacDonald, I.A., and M.J. Kuehn. (2012). Offense and defense: microbial membrane vesicles play both ways. *Research in microbiology,* 163:607-618.

Mahoney, J., C. Ferguson, M. Buchholtz, and C. Van Slyke. (1943). The Use of Penicillin Sodium in the Treatment of Sulfonamide. Resistant Gonorrhoea in Men. A Preliminary Report. *American Journal of Syphilis,* 27:525-528.

Mariani, L., and A. Venuti. (2010). HPV vaccine: an overview of immune response, clinical protection, and new approaches for the future. *Journal of Translational Medicine,* 8:105-105.

Martin, J.E., Jr., A. Lester, E.V. Price, and J.D. Schmale. (1970). Comparative study of gonococcal susceptibility to penicillin in the United States, 1955-1969. *The Journal of Infectious Diseases,* 122:459-461.

Mashburn, L.M., and M. Whiteley. (2005). Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature,* 437:422-425.

Massari, P., A. Visintin, J. Gunawardana, K.A. Halmen, C.A. King, D.T. Golenbock, and L.M. Wetzler. (2006). Meningococcal porin PorB binds to TLR2 and requires TLR1 for signaling. *The Journal of Immunology,* 176:2373-2380.

Mayfield, M. (1989). 1989 Sexually Transmitted Diseases Treatment Guidelines. U.S.D.o.H.a.H. Services, editor Center for Surveillance, Epidemiology, and Laboratory Services, Atlanta. 1-43.

McCaw, S.E., E.H. Liao, and S.D. Gray-Owen. (2004). Engulfment of Neisseria gonorrhoeae: revealing distinct processes of bacterial entry by individual carcinoembryonic antigen-related cellular adhesion molecule family receptors. *Infection and Immunity,* 72:2742-2752.

McCaw, S.E., J. Schneider, E.H. Liao, W. Zimmermann, and S.D. Gray-Owen. (2003). Immunoreceptor tyrosine-based activation motif phosphorylation during engulfment of Neisseria gonorrhoeae by the neutrophil-restricted CEACAM3 (CD66d) receptor. *Molecular Microbiology,* 49:623-637.

McChesney, D., E.C. Tramont, J.W. Boslego, J. Ciak, J. Sadoff, and C.C. Brinton. (1982). Genital antibody response to a parenteral gonococcal pilus vaccine. *Infection and immunity,* 36:1006-1012.

McCormack, W.M., K. Nowroozi, S. Alpert, S.G. Sackel, Y.H. Lee, E.W. Lowe, and J.S. Rankin. (1977a). Acute pelvic inflammatory disease: characteristics of patients with gonococcal and nongonococcal infection and evaluation of their response to treatment with aqueous procaine penicillin G and spectinomycin hydrochloride. *Sexually transmitted diseases,* 4:125-131.

McCormack, W.M., R.J. Stumacher, K. Johnson, and A. Donner. (1977b). Clinical spectrum of gonococcal infection in women. *Lancet,* 1:1182-1185.

McMahon, K.J., M.E. Castelli, E. Garcia Vescovi, and M.F. Feldman. (2012). Biogenesis of outer membrane vesicles in Serratia marcescens is thermoregulated and can be induced by activation of the Rcs phosphorelay system. *Journal of bacteriology,* 194:3241-3249.

Mestecky, J., and P.N. Fultz. (1999). Mucosal Immune System of the Human Genital Tract. *The Journal of Infectious Diseases,* 179:S470-S474.

Meyer, T.F., and J.P. van Putten. (1989). Genetic mechanisms and biological implications of phase variation in pathogenic neisseriae. *Clinical microbiology reviews,* 2:S139-145.

Molander, P., P. Finne, J. Sjoberg, J. Sellors, and J. Paavonen. (2003). Observer agreement with laparoscopic diagnosis of pelvic inflammatory disease using photographs. *Obstetrics and gynecology,* 101:875-880.

Muenzner, P., V. Bachmann, W. Zimmermann, J. Hentschel, and C.R. Hauck. (2010). Human-restricted bacterial pathogens block shedding of epithelial cells by stimulating integrin activation. *Science,* 329:1197-1201.

Muenzner, P., C. Dehio, T. Fujiwara, M. Achtman, T.F. Meyer, and S.D. Gray-Owen. (2000). Carcinoembryonic antigen family receptor specificity of Neisseria meningitidis Opa variants influences adherence to and invasion of proinflammatory cytokine-activated endothelial cells. *Infection and immunity,* 68:3601-3607.

Muenzner, P., M. Rohde, S. Kneitz, and C.R. Hauck. (2005). CEACAM engagement by human pathogens enhances cell adhesion and counteracts bacteria-induced detachment of epithelial cells. *The Journal of cell biology,* 170:825-836.

Muller, W.A., S.A. Weigl, X. Deng, and D.M. Phillips. (1993). PECAM-1 is required for transendothelial migration of leukocytes. *The Journal of experimental medicine.,* 178:449-460.

Muralidharan, K., A. Stern, and T.F. Meyer. (1987). The control mechanism of opacity protein expression in the pathogenic Neisseriae. *Antonie Van Leeuwenhoek,* 53:435-440.

Nardelli-Haefliger, D., R. Roden, C. Balmelli, A. Potts, J. Schiller, and P. De Grandi. (1999). Mucosal but not parenteral immunization with purified human papillomavirus type 16 virus-like particles induces neutralizing titers of antibodies throughout the estrous cycle of mice. *Journal of virology,* 73:9609-9613.

Ndowa, F.J., J.M. Francis, A. Machiha, H. Faye-Kette, and M.C. Fonkoua. (2013). Gonococcal antimicrobial resistance: perspectives from the African region. *Sexually transmitted infections,* 89 Suppl 4:iv11-15.

Neisser, A. (1879). Ueber eine der Gonorrhoe eigentümliche Micrococcusform: vorläufige Mitteilung.

Neisser, A. (1882). Die Mikrokokken der Gonorrhoe. *Deutsche Medizinische Wochenschrift,* 279-283.

Ngampasutadol, J., S. Ram, A.M. Blom, H. Jarva, A.E. Jerse, E. Lien, J. Goguen, S. Gulati, and P.A. Rice. (2005). Human C4b-binding protein selectively interacts with Neisseria gonorrhoeae and results in species-specific infection. *Proceedings*

of the National Academy of Sciences of the United States of America, 102:17142-17147.

Ngampasutadol, J., S. Ram, S. Gulati, S. Agarwal, C. Li, A. Visintin, B. Monks, G. Madico, and P.A. Rice. (2008). Human factor H interacts selectively with Neisseria gonorrhoeae and results in species-specific complement evasion. *The Journal of Immunology,* 180:3426-3435.

Ngampasutadol, J., P.A. Rice, M.T. Walsh, and S. Gulati. (2006). Characterization of a peptide vaccine candidate mimicking an oligosaccharide epitope of Neisseria gonorrhoeae and resultant immune responses and function. *Vaccine,* 24:157-170.

Nicolete, R., D.F.d. Santos, and L.H. Faccioli. (2011). The uptake of PLGA micro or nanoparticles by macrophages provokes distinct in vitro inflammatory response. *International Immunopharmacology,* 11:1557-1563.

Ohnishi, M., D. Golparian, K. Shimuta, T. Saika, S. Hoshina, K. Iwasaku, S. Nakayama, J. Kitawaki, and M. Unemo. (2011). Is Neisseria gonorrhoeae initiating a future era of untreatable gonorrhea?: detailed characterization of the first strain with high-level resistance to ceftriaxone. *Antimicrobial agents and chemotherapy,* 55:3538-3545.

Oliveira-Nascimento, L., P. Massari, and L.M. Wetzler. (2012). The Role of TLR2 in Infection and Immunity. *Frontiers in Immunology,* 3:79.

Olofsson, A., A. Vallstrom, K. Petzold, N. Tegtmeyer, J. Schleucher, S. Carlsson, R. Haas, S. Backert, S.N. Wai, G. Grobner, and A. Arnqvist. (2010). Biochemical and functional characterization of Helicobacter pylori vesicles. *Molecular Microbiology,* 77:1539-1555.

Oriel, J.D. (1994). The Scars of Venus: A History of Venereology. Springer-Verlag,

Osborne, N.G., and L. Grubin. (1979). Colonization of the pharynx with Neisseria gonorrhoeae: experience in a clinic for sexually transmitted diseases. *Sexually transmitted diseases,* 6:253-256.

Packiam, M., S.J. Veit, D.J. Anderson, R.R. Ingalls, and A.E. Jerse. (2010). Mouse strain-dependent differences in susceptibility to Neisseria gonorrhoeae infection and induction of innate immune responses. *Infection and immunity,* 78:433-440.

Pajon, R., A.M. Fergus, and D.M. Granoff. (2013). Mutant Native Outer Membrane Vesicles Combined with a Serogroup A Polysaccharide Conjugate Vaccine for Prevention of Meningococcal Epidemics in Africa. *PLoS One,* 8:e66536.

Palmer, H.M., H. Young, A. Winter, and J. Dave. (2008). Emergence and spread of azithromycin-resistant Neisseria gonorrhoeae in Scotland. *The Journal of antimicrobial chemotherapy,* 62:490-494.

Parr, E.L., and M.B. Parr. (1997). Immunoglobulin G is the main protective antibody in mouse vaginal secretions after vaginal immunization with attenuated herpes simplex virus type 2. *Journal of virology,* 71:8109-8115.

Parr, E.L., M.B. Parr, and M. Thapar. (1988). A comparison of specific antibody responses in mouse vaginal fluid after immunization by several routes. *Journal of reproductive immunology,* 14:165-176.

Pathirana, R.D., and M. Kaparakis-Liaskos. (2016). Bacterial membrane vesicles: Biogenesis, immune regulation and pathogenesis. *Cellular microbiology,* 18:1518-1524.

Patton, M.E., S. Kidd, E. Llata, M. Stenger, J. Braxton, L. Asbel, K. Bernstein, B. Gratzer, M. Jespersen, R. Kerani, C. Mettenbrink, M. Mohamed, P. Pathela, C. Schumacher, A. Stirland, J. Stover, I. Tabidze, R.D. Kirkcaldy, and H. Weinstock. (2014). Extragenital gonorrhea and chlamydia testing and infection among men who have sex with men--STD Surveillance Network, United States, 2010-2012. *Clinical infectious diseases,* 58:1564-1570.

Peipert, J.F., R.B. Ness, J. Blume, D.E. Soper, R. Holley, H. Randall, R.L. Sweet, S.J. Sondheimer, S.L. Hendrix, A. Amortegui, G. Trucco, D.C. Bass, E. Pelvic Inflammatory Disease, and I. Clinical Health Study. (2001). Clinical predictors of endometritis in women with symptoms and signs of pelvic inflammatory disease. *American journal of obstetrics and gynecology,* 184:856-863; discussion 863-854.

Percival, A., J. Rowlands, J.E. Corkill, C.D. Alergant, O.P. Arya, E. Rees, and E.H. Annels. (1976). Penicillinase-producing Gonococci in Liverpool. *Lancet,* 2:1379-1382.

Petousis-Harris, H., J. Paynter, J. Morgan, P. Saxton, B. McArdle, F. Goodyear-Smith, and S. Black. Effectiveness of a group B outer membrane vesicle meningococcal vaccine against gonorrhoea in New Zealand: a retrospective case-control study. *The Lancet,* 390:1603-1610.

Pettit, R.K., and R.C. Judd. (1992). The interaction of naturally elaborated blebs from serum-susceptible and serum-resistant strains of Neisseria gonorrhoeae with normal human serum. *Molecular Microbiology,* 6:729-734.

Phillips, I. (1976). Beta-lactamase-producing, penicillin-resistant gonococcus. *Lancet,* 2:656-657.

Plante, M., A. Jerse, J. Hamel, F. Couture, C.R. Rioux, B.R. Brodeur, and D. Martin. (2000). Intranasal immunization with gonococcal outer membrane preparations reduces the duration of vaginal colonization of mice by Neisseria gonorrhoeae. *The Journal of Infectious Diseases,* 182:848-855.

Platt, A., H. MacLeod, P. Massari, X. Liu, and L. Wetzler. (2013). In vivo and in vitro characterization of the immune stimulating activity of the Neisserial porin PorB. *PLoS One,* 8:e82171.

Plotkin, S., J.M. Robinson, G. Cunningham, R. Iqbal, and S. Larsen. (2017). The complexity and cost of vaccine manufacturing – An overview. *Vaccine,* 35:4064-4071.

Plummer, F.A., H. Chubb, J.N. Simonsen, M. Bosire, L. Slaney, I. Maclean, J.O. Ndinya-Achola, P. Waiyaki, and R.C. Brunham. (1993). Antibody to Rmp (outer membrane protein 3) increases susceptibility to gonococcal infection. *The Journal of clinical investigation,* 91:339-343.

Popp, A., C. Dehio, F. Grunert, T.F. Meyer, and S.D. Gray-Owen. (1999). Molecular analysis of neisserial Opa protein interactions with the CEA family of receptors: identification of determinants contributing to the differential specificities of binding. *Cellular microbiology,* 1:169-181.

Post, D.M., D. Zhang, J.S. Eastvold, A. Teghanemt, B.W. Gibson, and J.P. Weiss. (2005). Biochemical and functional characterization of membrane blebs purified from Neisseria meningitidis serogroup B. *The Journal of biological chemistry,* 280:38383-38394.

Powers, K., G. Lazarou, W.M. Greston, and M. Mikhail. (2007). Rupture of a tuboovarian abscess into the anterior abdominal wall: a case report. *The Journal of reproductive medicine,* 52:235-237.

Prevention, C.F.D.C.a. (2016). Sexually Transmitted Disease Surveillance 2015. U.S.D.o.H.a.H. Services, editor Atlanta.

Price, G.A., H.P. Masri, A.M. Hollander, M.W. Russell, and C.N. Cornelissen. (2007). Gonococcal transferrin binding protein chimeras induce bactericidal and growth inhibitory antibodies in mice. *Vaccine,* 25:7247-7260.

Qiu, H., Y. Fan, A.G. Joyee, S. Wang, X. Han, H. Bai, L. Jiao, N. Van Rooijen, and X. Yang. (2008). Type I IFNs enhance susceptibility to Chlamydia muridarum lung infection by enhancing apoptosis of local macrophages. *The Journal of Immunology,* 181:2092-2102.

Radtke, A.L., A.J. Quayle, and M.M. Herbst-Kralovetz. (2012). Microbial products alter the expression of membrane-associated mucin and antimicrobial peptides in a three-dimensional human endocervical epithelial cell model. *Biology of reproduction,* 87:132.

Ram, S., D.P. McQuillen, S. Gulati, C. Elkins, M.K. Pangburn, and P.A. Rice. (1998a). Binding of complement factor H to loop 5 of porin protein 1A: a molecular mechanism of serum resistance of nonsialylated Neisseria gonorrhoeae. *The Journal of experimental medicine.,* 188:671-680.

Ram, S., A.K. Sharma, S.D. Simpson, S. Gulati, D.P. McQuillen, M.K. Pangburn, and P.A. Rice. (1998b). A novel sialic acid binding site on factor H mediates serum resistance of sialylated Neisseria gonorrhoeae. *The Journal of experimental medicine.,* 187:743-752.

Ramani, K., S. Pawaria, K. Maers, A.R. Huppler, S.L. Gaffen, and P.S. Biswas. (2014). An essential role of interleukin-17 receptor signaling in the development of autoimmune glomerulonephritis. *Journal of leukocyte biology,* 96:463-472.

Ramsey, K.H., H. Schneider, R.A. Kuschner, A.F. Trofa, A.S. Cross, and C.D. Deal. (1994). Inflammatory cytokine response to experimental human infection with Neisseria gonorrhoeae. *Annals of the New York Academy of Sciences,* 730:322-325.

Ravindran, R., L. Rusch, A. Itano, M.K. Jenkins, and S.J. McSorley. (2007). CCR6-dependent recruitment of blood phagocytes is necessary for rapid CD4 T cell responses to local bacterial infection. *Proceedings of the National Academy of Sciences of the United States of America,* 104:12075-12080.

Reiser, M.L., M.M. Mosaheb, C. Lisk, A. Platt, and L.M. Wetzler. (2017). The TLR2 Binding Neisserial Porin PorB Enhances Antigen Presenting Cell Trafficking and Cross-presentation. *Scientific reports,* 7:736.

Rejnek, J., J. Trávníček, J. Kostka, J. Šterzl, and A. Lanc. (1968). Study of the effect of antibodies in the intestinal tract of germ-free baby pigs. *Folia Microbiologica,* 13:36-42.

Reyn, A., B. Korner, and M.W. Bentzon. (1958). Effects of penicillin, streptomycin, and tetracycline on N. gonorrhoeae isolated in 1944 and in 1957. *The British journal of venereal diseases,* 34:227-239.

Rice, P.A., E.W. Hook, M.S. Blake, R.S. Kaslow, S. Gulati, P.K. Kohl, M. VanRadden, and T.M. Buchanan. (1994). A possible influence of vaccine induced Por, LOS, and Rmp antibodies on the outcome of intraurethral challenge with Neisseria

gonorrhoeae. *Proceedings of the Ninth International Pathogenic Neisseria Conference,* 9:483-484.

Ritter, J.L., and C.A. Genco. (2018). Neisseria gonorrhoeae–Induced Inflammatory Pyroptosis in Human Macrophages is Dependent on Intracellular Gonococci and Lipooligosaccharide. *Journal of Cell Death,* 11:1179066017750902.

Rivera, J., R.J. Cordero, A.S. Nakouzi, S. Frases, A. Nicola, and A. Casadevall. (2010). Bacillus anthracis produces membrane-derived vesicles containing biologically active toxins. *Proceedings of the National Academy of Sciences of the United States of America,* 107:19002-19007.

Rosario, G., G. Sachdeva, W.C. Okulicz, C.I. Ace, R.R. Katkam, and C.P. Puri. (2003). Role of progesterone in structural and biochemical remodeling of endometrium. *Frontiers in bioscience,* 8:s924-935.

Ross, J. (2015). Pelvic inflammatory disease: Pathogenesis, microbiology, and risk factors. Up To Date. Wolters Klewer.

Ross, J., P. Judlin, J. Jensen, and i. International Union against sexually transmitted. (2014). 2012 European guideline for the management of pelvic inflammatory disease. *International journal of STD & AIDS,* 25:1-7.

Rowley, J., I. Toskin, and F. Ndowa. (2012). Global incidence and prevalence of selected curable sexually transmitted infections – 2008. D.o.R.H.a. Research, editor World Health Organization.

Russell, M.W., and J. Mestecky. (2002). Humoral immune responses to microbial infections in the genital tract. *Microbes and Infection,* 4:667-677.

Sadarangani, M., A.J. Pollard, and S.D. Gray-Owen. (2011). Opa proteins and CEACAMs: pathways of immune engagement for pathogenic Neisseria. *FEMS microbiology reviews,* 35:498-514.

Satterwhite, C.L., E. Torrone, E. Meites, E.F. Dunne, R. Mahajan, M.C. Ocfemia, J. Su, F. Xu, and H. Weinstock. (2013). Sexually transmitted infections among US women and men: prevalence and incidence estimates, 2008. *Sexually transmitted diseases,* 40:187-193.

Schachter, J., J. Moncada, S. Liska, C. Shayevich, and J.D. Klausner. (2008). Nucleic acid amplification tests in the diagnosis of chlamydial and gonococcal infections of the oropharynx and rectum in men who have sex with men. *Sexually transmitted diseases,* 35:637-642.

Schiffmann, E., B.A. Corcoran, and S.M. Wahl. (1975). N-formylmethionyl peptides as chemoattractants for leucocytes. *Proceedings of the National Academy of Sciences of the United States of America,* 72:1059-1062.

Scurlock, A.M., L.C. Frazer, C.W. Andrews, Jr., C.M. O'Connell, I.P. Foote, S.L. Bailey, K. Chandra-Kuntal, J.K. Kolls, and T. Darville. (2011). Interleukin-17 contributes to generation of Th1 immunity and neutrophil recruitment during Chlamydia muridarum genital tract infection but is not required for macrophage influx or normal resolution of infection. *Infection and immunity,* 79:1349-1362.

Seifert, H.S., C.J. Wright, A.E. Jerse, M.S. Cohen, and J.G. Cannon. (1994). Multiple gonococcal pilin antigenic variants are produced during experimental human infections. *The Journal of clinical investigation,* 93:2744-2749.

Sherrard, J., and D. Barlow. (1996). Gonorrhoea in men: clinical and diagnostic aspects. *Genitourinary medicine,* 72:422-426.

Sherwani, M.A., S. Tufail, A.F. Muzaffar, and N. Yusuf. (2018). The skin microbiome and immune system: Potential target for chemoprevention? *Photodermatology, Photoimmunology & Photomedicine,* 34:25-34.

Sintsova, A., H. Sarantis, E.A. Islam, C.X. Sun, M. Amin, C.H. Chan, C.P. Stanners, M. Glogauer, and S.D. Gray-Owen. (2014). Global analysis of neutrophil responses to Neisseria gonorrhoeae reveals a self-propagating inflammatory program. *PLoS Pathogens,* 10:e1004341.

Song, W., S. Condron, B.T. Mocca, S.J. Veit, D. Hill, A. Abbas, and A.E. Jerse. (2008). Local and humoral immune responses against primary and repeat Neisseria gonorrhoeae genital tract infections of 17beta-estradiol-treated mice. *Vaccine,* 26:5741-5751.

Sonntag, I., H. Schwarz, Y. Hirota, and U. Henning. (1978). Cell envelope and shape of Escherichia coli: multiple mutants missing the outer membrane lipoprotein and other major outer membrane proteins. *Journal of bacteriology,* 136:280-285.

Soper, D.E., N.J. Brockwell, H.P. Dalton, and D. Johnson. (1994). Observations concerning the microbial etiology of acute salpingitis. *American journal of obstetrics and gynecology,* 170:1008-1014; discussion 1014-1007.

Staley, K., and H. Scharfman. (2005). A woman's prerogative. 697-699 pp.

Subramanian, A., H. Kuehn, J. Gould, P. Tamayo, and J.P. Mesirov. (2007). GSEA-P: a desktop application for Gene Set Enrichment Analysis. *Bioinformatics,* 23:3251-3253.

Subramanian, A., P. Tamayo, V.K. Mootha, S. Mukherjee, B.L. Ebert, M.A. Gillette, A. Paulovich, S.L. Pomeroy, T.R. Golub, E.S. Lander, and J.P. Mesirov. (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America,* 102:15545-15550.

Swanson, J. (1978). Studies on gonococcus infection. XIV. Cell wall protein differences among color/opacity colony variants of Neisseria gonorrhoeae. *Infection and Immunity,* 21:292-302.

Swanson, J. (1981). Surface-exposed protein antigens of the gonococcal outer membrane. *Infection and immunity,* 34:804-816.

Taylor-Robinson, D., P.M. Furr, and C.M. Hetherington. (1990). Neisseria gonorrhoeae colonises the genital tract of oestradiol-treated germ-free female mice. *Microbial pathogenesis,* 9:369-373.

Tramont, E.C. (1989). Gonococcal vaccines. *Clinical microbiology reviews,* 2 Suppl:S74-77.

Tregoning, J.S., V. Buffa, A. Oszmiana, K. Klein, A.A. Walters, and R.J. Shattock. (2013). A "prime-pull" vaccine strategy has a modest effect on local and systemic antibody responses to HIV gp140 in mice. *PLoS One,* 8:e80559.

Unemo, M., D. Golparian, and B. Hellmark. (2014). First three Neisseria gonorrhoeae isolates with high-level resistance to azithromycin in Sweden: a threat to currently available dual-antimicrobial regimens for treatment of gonorrhea? *Antimicrobial agents and chemotherapy,* 58:624-625.

Unemo, M., D. Golparian, R. Nicholas, M. Ohnishi, A. Gallay, and P. Sednaoui. (2012). High-level cefixime- and ceftriaxone-resistant Neisseria gonorrhoeae in France: novel penA mosaic allele in a successful international clone causes treatment failure. *Antimicrobial agents and chemotherapy,* 56:1273-1280.

Unemo, M., D. Golparian, A. Stary, and A. Eigentler. (2011). First Neisseria gonorrhoeae strain with resistance to cefixime causing gonorrhoea treatment failure in Austria, 2011. *Euro surveillance : bulletin Européen sur les maladies transmissibles,* 16:

Unemo, M., and R.A. Nicholas. (2012). Emergence of multidrug-resistant, extensively drug-resistant and untreatable gonorrhea. *Future microbiology,* 7:1401-1422.

Unemo, M., and W.M. Shafer. (2011). Antibiotic resistance in Neisseria gonorrhoeae: origin, evolution, and lessons learned for the future. *Annals of the New York Academy of Sciences,* 1230:E19-28.

Unemo, M., and W.M. Shafer. (2014). Antimicrobial resistance in Neisseria gonorrhoeae in the 21st century: past, evolution, and future. *Clinical microbiology reviews,* 27:587-613.

Usala, S.J., F.O. Usala, R. Haciski, J.A. Holt, and G.F. Schumacher. (1989). IgG and IgA content of vaginal fluid during the menstrual cycle. *The Journal of reproductive medicine,* 34:292-294.

van de Waterbeemd, B., G.P. Mommen, J.L. Pennings, M.H. Eppink, R.H. Wijffels, L.A. van der Pol, and A.P. de Jong. (2013). Quantitative proteomics reveals distinct differences in the protein content of outer membrane vesicle vaccines. *Journal of proteome research,* 12:1898-1908.

van de Waterbeemd, B., M. Streefland, P. van der Ley, B. Zomer, H. van Dijken, D. Martens, R. Wijffels, and L. van der Pol. (2010). Improved OMV vaccine against Neisseria meningitidis using genetically engineered strains and a detergent-free purification process. *Vaccine,* 28:4810-4816.

van der Ley, P., L. Steeghs, H.J. Hamstra, J. ten Hove, B. Zomer, and L. van Alphen. (2001). Modification of lipid A biosynthesis in Neisseria meningitidis lpxL mutants: influence on lipopolysaccharide structure, toxicity, and adjuvant activity. *Infection and immunity,* 69:5981-5990.

van der Pol, L., M. Stork, and P. van der Ley. (2015). Outer membrane vesicles as platform vaccine technology. *Biotechnology Journal,* 10:1689-1706.

Van Slyke, C.J., R.C. Arnold, and M. Buchholtz. (1943). Penicillin Therapy in Sulfonamide-Resistant Gonorrhea in Men. *American journal of public health and the nation's health,* 33:1392-1394.

Vanaja, S.K., A.J. Russo, B. Behl, I. Banerjee, M. Yankova, S.D. Deshmukh, and V.A.K. Rathinam. (2016). Bacterial Outer Membrane Vesicles Mediate Cytosolic Localization of LPS and Caspase-11 Activation. *Cell,* 165:1106-1119.

Vaughn, D.E., and P.J. Bjorkman. (1996). The (Greek) key to structures of neural adhesion molecules. *Neuron,* 16:261-273.

Verma, V. (1983). Ultrastructural changes in human endometrium at different phases of the menstrual cycle and their functional significance. *Gynecologic and obstetric investigation,* 15:193-212.

Virji, M., D. Evans, A. Hadfield, F. Grunert, A.M. Teixeira, and S.M. Watt. (1999). Critical determinants of host receptor targeting by Neisseria meningitidis and Neisseria gonorrhoeae: identification of Opa adhesiotopes on the N-domain of CD66 molecules. *Molecular Microbiology,* 34:538-551.

Voges, M., V. Bachmann, R. Kammerer, U. Gophna, and C.R. Hauck. (2010). CEACAM1 recognition by bacterial pathogens is species-specific. *BioMed Central microbiology,* 10:117.

von Andrian, U.H., J.D. Chambers, E.L. Berg, S.A. Michie, D.A. Brown, D. Karolak, L. Ramezani, E.M. Berger, K.E. Arfors, and E.C. Butcher. (1993). L-selectin mediates neutrophil rolling in inflamed venules through sialyl LewisX-dependent and -independent recognition pathways. *Blood,* 82:182-191.

Wai, S.N., B. Lindmark, T. Soderblom, A. Takade, M. Westermark, J. Oscarsson, J. Jass, A. Richter-Dahlfors, Y. Mizunoe, and B.E. Uhlin. (2003). Vesicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ClyA cytotoxin. *Cell,* 115:25-35.

Wang, J., S.D. Gray-Owen, A. Knorre, T.F. Meyer, and C. Dehio. (1998). Opa binding to cellular CD66 receptors mediates the transcellular traversal of Neisseria gonorrhoeae across polarized T84 epithelial cell monolayers. *Molecular Microbiology,* 30:657-671.

Wang, Y. (2002). The function of OmpA in Escherichia coli. *Biochemical and biophysical research communications,* 292:396-401.

Wessel, F., M. Winderlich, M. Holm, M. Frye, R. Rivera-Galdos, M. Vockel, R. Linnepe, U. Ipe, A. Stadtmann, A. Zarbock, A.F. Nottebaum, and D. Vestweber. (2014). Leukocyte extravasation and vascular permeability are each controlled in vivo by different tyrosine residues of VE-cadherin. *Nature immunology,* 15:223-230.

West, S.E., and P.F. Sparling. (1985). Response of Neisseria gonorrhoeae to iron limitation: alterations in expression of membrane proteins without apparent siderophore production. *Infection and immunity,* 47:388-394.

Wetzler, L. (2014). The Roadblocks in Developing a Gonococcal Vaccine and Reasons for Optimism. In Pathogenic Neisseria. J.K. Davies, editor Caister Academic Press,

Wetzler, L.M. (2010). Innate immune function of the neisserial porins and the relationship to vaccine adjuvant activity. *Future microbiology,* 5:749-758.

Wetzler, L.M., K. Barry, M.S. Blake, and E.C. Gotschlich. (1992a). Gonococcal lipooligosaccharide sialylation prevents complement-dependent killing by immune sera. *Infection and immunity,* 60:39-43.

Wetzler, L.M., M.S. Blake, K. Barry, and E.C. Gotschlich. (1992b). Gonococcal porin vaccine evaluation: comparison of Por proteosomes, liposomes, and blebs isolated from rmp deletion mutants. *The Journal of Infectious Diseases,* 166:551-555.

Wetzler, L.M., M.S. Blake, and E.C. Gotschlich. (1988). Characterization and specificity of antibodies to protein I of Neisseria gonorrhoeae produced by injection with various protein I-adjuvant preparations. *The Journal of experimental medicine.,* 168:1883-1897.

Wetzler, L.M., E.C. Gotschlich, M.S. Blake, and J.M. Koomey. (1989). The construction and characterization of Neisseria gonorrhoeae lacking protein III in its outer membrane. *The Journal of experimental medicine.,* 169:2199-2209.

Wiesenfeld, H.C., R.L. Sweet, R.B. Ness, M.A. Krohn, A.J. Amortegui, and S.L. Hillier. (2005). Comparison of acute and subclinical pelvic inflammatory disease. *Sexually transmitted diseases,* 32:400-405.

Wiesner, P.J., E. Tronca, P. Bonin, A.H. Pedersen, and K.K. Holmes. (1973). Clinical spectrum of pharyngeal gonococcal infection. *The New England journal of medicine,* 288:181-185.

Willcox, R.R. (1970). A survey of problems in the antibiotic treatment of gonorrhoea. With special reference to South-East Asia. *The British journal of venereal diseases,* 46:217-242.

Williams, A.F., and A.N. Barclay. (1988). The immunoglobulin superfamily--domains for cell surface recognition. *Annual review of immunology,* 6:381-405.

Williams, D.A., W. Tao, F. Yang, C. Kim, Y. Gu, P. Mansfield, J.E. Levine, B. Petryniak, C.W. Derrow, C. Harris, B. Jia, Y. Zheng, D.R. Ambruso, J.B. Lowe, S.J. Atkinson, M.C. Dinauer, and L. Boxer. (2000). Dominant negative mutation of the hematopoietic-specific Rho GTPase, Rac2, is associated with a human phagocyte immunodeficiency. *Blood,* 96:1646-1654.

Winter, J., D. Letley, J. Rhead, J. Atherton, and K. Robinson. (2014). Helicobacter pylori membrane vesicles stimulate innate pro- and anti-inflammatory responses and induce apoptosis in Jurkat T cells. *Infection and immunity,* 82:1372-1381.

Workowski, K., and G. Bolan. (2015). Sexually Transmitted Diseases Treatment Guidelines, 2015. U.S.D.o.H.a.H. Services, editor Center for Surveillance, Epidemiology, and Laboratory Services, Atlanta. 60-68.

Workowski, K.A., G.A. Bolan, C. Centers for Disease, and Prevention. (2015). Sexually transmitted diseases treatment guidelines, 2015. *MMWR Recomm Rep,* 64:1-137.

Wu, Z., L. Xu, Y. Tu, R. Chen, Y. Yu, J. Li, M. Tan, and H. Chen. (2011). The relationship between the symptoms of female gonococcal infections and serum progesterone level and the genotypes of Neisseria gonorrhoeae multi-antigen

sequence type (NG-MAST) in Wuhan, China. *European journal of clinical microbiology & infectious diseases,* 30:113-116.

Xie, Q., J. Klesney-Tait, K. Keck, C. Parlet, N. Borcherding, R. Kolb, W. Li, L. Tygrett, T. Waldschmidt, A. Olivier, S. Chen, G.H. Liu, X. Li, and W. Zhang. (2015). Characterization of a novel mouse model with genetic deletion of CD177. *Protein and Cell,* 6:117-126.

Yamanishi, Y., M. Takahashi, K. Izawa, M. Isobe, S. Ito, A. Tsuchiya, A. Maehara, A. Kaitani, T. Uchida, K. Togami, Y. Enomoto, F. Nakahara, T. Oki, M. Kajikawa, H. Kurihara, T. Kitamura, and J. Kitaura. (2012). A soluble form of LMIR5/CD300b amplifies lipopolysaccharide-induced lethal inflammation in sepsis. *The Journal of Immunology,* 189:1773-1779.

Yamashita, K., K. Totani, Y. Iwaki, M. Kuroki, Y. Matsuoka, T. Endo, and A. Kobata. (1989). Carbohydrate structures of nonspecific cross-reacting antigen-2, a glycoprotein purified from meconium as an antigen cross-reacting with anticarcinoembryonic antigen antibody. Occurrence of complex-type sugar chains with the Gal beta 1----3GlcNAc beta 1----3Gal beta 1----4GlcNAc beta 1----outer chains. *The Journal of biological chemistry,* 264:17873-17881.

Yamashita, K., K. Totani, M. Kuroki, Y. Matsuoka, I. Ueda, and A. Kobata. (1987). Structural studies of the carbohydrate moieties of carcinoembryonic antigens. *Cancer research,* 47:3451-3459.

Yamazaki, T., X.O. Yang, Y. Chung, A. Fukunaga, R. Nurieva, B. Pappu, N. Martin-Orozco, H.S. Kang, L. Ma, A.D. Panopoulos, S. Craig, S.S. Watowich, A.M. Jetten, Q. Tian, and C. Dong. (2008). CCR6 regulates the migration of inflammatory and regulatory T cells. *The Journal of Immunology,* 181:8391-8401.

Yanez-Mo, M., P.R. Siljander, Z. Andreu, A.B. Zavec, F.E. Borras, E.I. Buzas, K. Buzas, E. Casal, F. Cappello, J. Carvalho, E. Colas, A. Cordeiro-da Silva, S. Fais, J.M. Falcon-Perez, I.M. Ghobrial, B. Giebel, M. Gimona, M. Graner, I. Gursel, M. Gursel, N.H. Heegaard, A. Hendrix, P. Kierulf, K. Kokubun, M. Kosanovic, V. Kralj-Iglic, E.M. Kramer-Albers, S. Laitinen, C. Lasser, T. Lener, E. Ligeti, A. Line, G. Lipps, A. Llorente, J. Lotvall, M. Mancek-Keber, A. Marcilla, M. Mittelbrunn, I. Nazarenko, E.N. Nolte-'t Hoen, et al. (2015). Biological properties of extracellular vesicles and their physiological functions. *Journal of extracellular vesicles,* 4:27066.

Yang, L., R.M. Froio, T.E. Sciuto, A.M. Dvorak, R. Alon, and F.W. Luscinskas. (2005). ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-alpha-activated vascular endothelium under flow. *Blood,* 106:584-592.

Yang, Q.L., and E.C. Gotschlich. (1996). Variation of gonococcal lipooligosaccharide structure is due to alterations in poly-G tracts in lgt genes encoding glycosyl transferases. *The Journal of experimental medicine*., 183:323-327.

Yokoyama, K., T. Horii, T. Yamashino, S. Hashikawa, S. Barua, T. Hasegawa, H. Watanabe, and M. Ohta. (2000). Production of shiga toxin by Escherichia coli measured with reference to the membrane vesicle-associated toxins. *FEMS microbiology letters,* 192:139-144.

You, J.S., M.J. Kim, H.S. Chung, Y.E. Chung, I. Park, S.P. Chung, S. Kim, and H.S. Lee. (2012). Clinical features of Fitz-Hugh-Curtis Syndrome in the emergency department. *Yonsei medical journal,* 53:753-758.

Youssef, A.R., M. van der Flier, S. Estevao, N.G. Hartwig, P. van der Ley, and M. Virji. (2009). Opa+ and Opa- isolates of Neisseria meningitidis and Neisseria gonorrhoeae induce sustained proliferative responses in human CD4+ T cells. *Infection and immunity,* 77:5170-5180.

Zhu, W., C.J. Chen, C.E. Thomas, J.E. Anderson, A.E. Jerse, and P.F. Sparling. (2011). Vaccines for gonorrhea: can we rise to the challenge? *Frontiers in microbiology,* 2:124.

Zhu, W., C.E. Thomas, C.J. Chen, C.N. Van Dam, R.E. Johnston, N.L. Davis, and P.F. Sparling. (2005). Comparison of immune responses to gonococcal PorB delivered as outer membrane vesicles, recombinant protein, or Venezuelan equine encephalitis virus replicon particles. *Infection and immunity,* 73:7558-7568.

Zielke, R.A., I.H. Wierzbicki, J.V. Weber, P.R. Gafken, and A.E. Sikora. (2014). Quantitative proteomics of the Neisseria gonorrhoeae cell envelope and membrane vesicles for the discovery of potential therapeutic targets. *Molecular & cellular proteomics,* 13:1299-1317.

Curriculum Vitae