Neutrophilic inflammation initiated by gonococcal-endocervical cell interactions and amplified by migrating neutrophils

Jacqueline Suzanne Stevens
South Burlington, Vermont

Bachelor of Science in Biochemistry, the University of Virginia, 2012

A dissertation presented to the Graduate Faculty
of the University of Virginia in Candidacy for
the Degree of Doctor of Philosophy

Department of Microbiology, Immunology and Cancer Biology

University of Virginia
August, 2018

_____

_____

_____

_____

_____

Abstract

The Gram-negative diplococcus *Neisseria gonorrhoeae* causes the prevalent sexually transmitted infection, gonorrhea. Gonorrhea is a major global health concern, with over 78 million infections world-wide per year. Of particular concern are the lack of a lasting immune response, the lack of a vaccine, and the rise in antimicrobial resistance, with some strains resistant to multiple antibiotics. Female infection is often asymptomatic, and as such, women are particularly at risk for severe clinical consequences including pelvic inflammatory disease, ectopic pregnancy, and infertility.

Infection with the human-specific pathogen *Neisseria gonorrhoeae* (Gc) is characterized by robust neutrophil influx that is insufficient to clear Gc. While infected men are typically symptomatic and seek treatment, female infection is often asymptomatic. Importantly, women have neutrophilic cervicitis whether or not they notice symptoms. Sustained infection and neutrophilic inflammation contribute to host cell damage and serious clinical sequelae in women including pelvic inflammatory disease and infertility. While previous work has shown that Gc interacts with epithelial cells through various surface-exposed features, the specific Gc-cervical cell interactions that stimulate neutrophil transepithelial migration were unknown. Previous models used to study Gc infection do not incorporate neutrophils and do not completely mimic initial infection and neutrophil influx at the cervix. Therefore, to investigate this site-specific neutrophilic inflammation, we established a three-component gonococcal-endocervical epithelial cell-neutrophil co-culture system to investigate neutrophil influx following infection. Using this system, we find that polarized End1 monolayers support apical infection with Gc and that primary human neutrophils migrate from the basal-to-apical direction following apical infection. Neutrophil transepithelial migration required both an epithelial cell contribution and bacterial-epithelial cell contact.

Bioactive lipids derived from arachidonic acid (AA) by lipoxygenases include leukotriene B_4 (LTB_4) and hepoxilin A_3 (HXA_3) and are potent neutrophil chemoattractants. Chemical inhibition of epithelial PKC and $cPLA_2$ that lead to AA release inhibited neutrophil transepithelial migration to Gc. Neutrophil transepithelial migration did not require epithelial 5-lipoxygenase activity but was amplified by neutrophil 5-lipoxygenase-dependent production of LTB_4. Pharmacologic and genetic targeting of endocervical 12-lipoxygenase activity inhibited neutrophil transepithelial migration to Gc. Additionally, neutrophil transepithelial migration was abrogated by selective inhibition of MRP2 channels that transport HXA_3 and by addition of recombinant human soluble epoxide hydrolase (sEH) that specifically degrades HXA_3. Further, trypsin-shaved Gc stimulated significantly less neutrophil transepithelial migration. Our work supports a model in which Gc-endocervical cell contact stimulates epithelial signaling events leading to 12-lipoxygenase activation, HXA_3 production, and consequent neutrophil transepithelial migration that is amplified by neutrophil-derived LTB_4. These studies are the first to define the molecular events that initiate and drive neutrophilic inflammation in the context of physiologically relevant cervical infection by Gc. This work reveals novel adjunctive therapeutic targets for limiting the deleterious sequelae associated with neutrophilic inflammation to gonorrhea in women.

Acknowledgements

I am indebted to a number of people who have helped and supported me throughout my dissertation work. I want to thank my mentor Alison Criss, for her ever-insightful guidance, feedback, and discussion throughout our time together. Our conversations, in passing and in meetings provided me the framework for thinking deeply about my project, asking questions, and evaluating progress and gaps in my work. Alison has also served as an invaluable mentor in terms of career development, and I have valued our conversations about women in science and medicine and career goals in general. I have really enjoyed working with Alison!

I would also like to thank the members of my thesis committee, Loren Erickson, Jim Casanova, Bill Petri, and Isabelle Derré. Our conversations have been incredibly fruitful. I always came away from committee meetings re-invigorated and with fresh ideas and insight into my work. I have also valued the conversations we have had outside of our formal meetings, and I would not be where I am today without their mentorship and guidance. Their support of my progress, both in my research and professionally at conferences and presentations, has been invaluable to my training experience.

I would not be where I am today without the wonderful present and past members of the Criss laboratory. These members include Asya Smirnov, Mary Gray, Stephanie Ragland, Brittany Johnson, Richard Juneau, Jonathan Handing, Kylene Daily, Louise Ball, Allie Palmer, Jocelyn Ray, Lacie Werner, Amaris Caderas, Katie Hill, Urmila Bharathan, Rebecca Dunning, Sam Clark, and Rhea Kapania. I want to thank each and every one of them for their continued support and encouragement throughout my graduate work. I would like to give special thanks to Stephanie Ragland, a well-organized and incredibly bright graduate student colleague and travel partner. I would also like to give special thanks to Asya Smirnov, who has been a constant friend and

mentor for me – our conversations have spanned from scientific and intellectual to life and career topics and have contributed incredible richness to my graduate years!

My graduate experience would not have been complete without the many friends I have made along the way. These include fellow MSTP students and BIMS students, as well as graduate students from other departments across grounds. Whether through involvement in the MSTP, the Raven Society, Advocates for Females in Research and Medicine (AFFIRM), or in informal occasions outside of school, the friends I have gathered have enriched my UVA experience in immeasurable ways. Outside of UVA, my long-time friends, both from high school and college have been supportive and present in my life, and I could not do without them.

Finally I would like to thank my family – especially my Mom (Nanette), Dad (Bob), and two sisters (Jenn and Nina), who are ever present to help and guide me. They have provided encouragement, council, and endless support throughout my graduate years. I would not be the student, friend, or person I am today without them! My family includes my Charlottesville friends, who I have gathered over the years and who have provided countless opportunities for fun and growth through engagement in our community, outdoor adventures, and fun. My family also includes my dog Lilly. Anyone who has known me since getting Lilly at the beginning of graduate school knows that she is the center of my world. She provides me endless love, cuddles, and adventures and is an ever-calming and happy presence in my life. Thank you so very much to everyone!

Table of Contents

Table of Figures

List of Tables

1. Chapter 1: Introduction

This chapter contains parts from the previously published article, “Pathogenesis of Neisseria gonorrhoeae in the female reproductive tract: Neutrophilic host response, sustained infection, and clinical sequelae” [Curr Opin Hematol. 2018 Jan;25(1):13-21. doi: 10.1097/MOH.0000000000000394]. https://journals.lww.com/co-hematology/Abstract/2018/01000/Pathogenesis_of_Neisseria_gonorrhoeae_in_the.5.aspx

1.1 Gonorrheal disease is a major global health concern

The Gram-negative diplococcus *Neisseria gonorrhoeae* (the gonococcus or Gc) causes the sexually transmitted infection gonorrhea, with an estimated 78 million cases worldwide each year (Wi et al., 2017). Increasing rates of infection, the recent emergence of strains that are resistant to most or all clinically recommended antibiotics, and the inability to develop a protective vaccine despite decades of research, have prompted the CDC and WHO to classify Gc as a threat level urgent pathogen (Wi et al., 2017). Women are particularly at risk for negative outcomes associated with gonorrhea, including pelvic inflammatory disease (PID), tubal scarring, ectopic pregnancy, and infertility (Mayor et al., 2012).

Infection elicits a robust inflammatory response featuring the influx of neutrophils (polymorphonuclear leukocytes or PMNs). Despite this potent immune response, secretions from infected individuals contain viable bacteria, many of which are associated with neutrophils (Johnson and Criss, 2011; Wiesner and Thompson, 1980) (Figure 1). Sustained neutrophil influx in other infectious conditions has been linked to epithelial cell damage and pathology associated with disease (Kruger et al., 2015; Porto and Stein, 2016; Williams and Parkos, 2007). We posit that the ability of Gc to elicit

neutrophilic inflammation in the female reproductive tract (FRT) is central to its ability to persist within its obligate human host and be transmitted to new hosts, while contributing to negative outcomes in women.

1.2 Gonococcal infection in the female reproductive tract

1.2.1 *Differences between male and female Gc infection*

Gonorrhea presents most commonly as urethritis in men and cervicitis in women, however Gc can also infect the oropharynx, rectum, and conjunctiva, and rarely can cause disseminated infection (Wiesner and Thompson, 1980). In men, infection is usually symptomatic, presenting as a purulent urethral discharge containing neutrophils, Gc, and shed urethral epithelial cells (Edwards and Apicella, 2004). In contrast, initial female infection is most often asymptomatic (Edwards and Apicella, 2004), meaning that infected women do not notice symptoms and/or seek treatment. This is particularly problematic because untreated female infection can ascend into the upper FRT, where continued infection and the resulting inflammatory response contribute to significant tissue damage that can lead to PID, tubal scaring, infertility, and ectopic pregnancy (Edwards and Apicella, 2004).

1.2.2 *Cervical epithelial cells coordinate initial defenses against Gc infection in the female reproductive tract*

The initial site of Gc infection in women is the endocervix, an area of transition from the lower FRT, which has a resident microbiota, compared to the upper FRT, which is largely considered to be sterile (Figure 2). The endocervix also marks a transition from

multilayered squamous epithelium in the lower FRT to single columnar epithelium lining the upper FRT (Hickey et al., 2011; Yarbrough et al., 2015). Cervical epithelial cells coordinate initial defenses against Gc and other invading pathogens in the FRT. Mucosal epithelial defenses at the level of the cervix include epithelial cell tight junctions, production of antimicrobial peptides (AMPs) in high local concentrations, and thick cervical mucus (Quayle, 2002). The majority of these defenses are influenced by hormones in the FRT, including estrogen, as well as inflammatory biomarkers and invading pathogens and/or changes in the microbiota (Yarbrough et al., 2015). Tight junctional complexes between cervical epithelial cells maintain barrier function against invading pathogens, inhibiting their paracellular movement into the subepithelial space (Blaskewicz et al., 2011; Yarbrough et al., 2015). Cervical epithelial cells secrete antimicrobials including defensins and lysozyme, which accumulate in high concentrations in cervical mucus and can help kill invading pathogens (Hickey et al., 2011; Yarbrough et al., 2015). Cationic AMPs (CAMPs) target microbes by associating with the negatively charged surface of bacterial membranes (Yarbrough et al., 2015) as well as via enzymatic activity as seen in the case of lysozyme (Ragland and Criss, 2017). CAMP secretion by FRT epithelial cells is influenced by the microbiota as well as in response to invading pathogens (Yarbrough et al., 2015). Mucus, which is comprised of glycosylated mucin proteins, helps to trap pathogens and impede their movement through the cervix and serves as a site of concentrated CAMPs (Hickey et al., 2011). Additionally, commensal microbiota, most commonly *Lactobacillus* in the lower female reproductive tract produce lactic acid to maintain an acidic pH that blunts growth of pathogenic bacteria and produce factors that are active against pathogenic bacteria, including hydrogen peroxide (Martin et al., 2008; Quayle, 2002; Witkin et al., 2007).

Epithelial and sentinel immune cells in the FRT express both intracellular and extracellular pattern recognition receptors (PRRs) that help initiate the immune response

to pathogens. Mucosal epithelial responsiveness and myeloid cell frequencies are titrated based on location in the FRT, with epithelial cells in particular becoming more responsive to pathogen-associated molecular patterns (PAMPs) moving up the reproductive tract (Figure 2). Additionally, the proportion of resident myeloid cells increases moving from the lower to the upper FRT (Givan et al., 1997). Further, neutrophils are not present in the tissue unless they are recruited from the bloodstream by gradients of inflammatory mediators (Borregaard, 2010). This ensures that a robust neutrophilic inflammatory response is only initiated for pathogens that attach to and invade the endocervix or upper FRT but not to resident microbiota of the lower FRT. Therefore, the cervix is an important transitional site between the relatively immune-tolerant lower FRT and the immune-responsive upper FRT.

1.2.3 *Initial Gc-cervical epithelial cell interactions are mediated by prominent Gc surface factors*

Gc interacts with host epithelial cells via surface-exposed features including opacity-associated (Opa) proteins, type IV pili, lipooligosaccharide (LOS), and porin (Edwards and Apicella, 2004; Edwards and Butler, 2011; Merz and So, 2000). Gc can antigenically vary expression of the type IV pilus, Opa proteins, and LOS (Virji, 2009), which aids in immune evasion and influences host-cell interactions during infection.

In Gc, the type IV pilus is involved in Gc interactions with host cells and contributes to immune evasion via phase and antigenic variation. The importance of the type IV pilus during infection is highlighted by the observation that Gc recovered from infected patients (male and female) as well as from the experimental male challenge model are piliated (Kellogg et al., 1968; Kellogg et al., 1963). The major pilin subunit, PilE, exhibits high-frequency antigenic variation via recombination of *pilE* with one of

many potential silent pilin, *pilS* genes (Cahoon and Seifert, 2011; Haas and Meyer, 1986). The type IV pilus also exhibits ON/OFF phase variation mediated either by expression of PilC or via recombination of *pilE* that results in a non-expressed major pilin subunit. PilC is located on the tip of the pilus and contributes to host cell adhesion, regulates type IV pilus retraction by countering the activity of PilT, the pilin subunit that exerts a retractile force on the type IV pilus to promote pilus retraction, and is required for expression of the pilus (Morand et al., 2004; Rudel et al., 1995; Rudel et al., 1992). The type IV pilus is extruded through a pore formed by PilQ, and loss of *pilQ* results in non-piliated Gc (Drake and Koomey, 1995). Piliated Gc are more resistant to ROS and neutrophil-mediated killing *in vitro* (Stohl et al., 2013), which supports the observation that piliated Gc are selected for *in vivo* (Kellogg et al., 1968; Kellogg et al., 1963). Therefore, the type IV pilus contributes to host cell interactions as an adhesin, may contribute to survival in the presence of neutrophils, and helps Gc evade a protective antibody-mediated immune response via phase and antigenic variation.

Studies in immortalized and primary epithelial cells support a role for the type IV pilus in mediating initial attachment to host epithelial cells (reviewed in (Merz and So, 2000)). In addition, the type IV pilus helps mediate Gc aggregation, twitching motility, and natural DNA transformation (Cahoon and Seifert, 2011). The PilT component of the type IV pilus confers twitching motility on Gc (Merz et al., 2000). Type IV pili exert a retractile force that stimulates actin cytoskeletal rearrangements, cortical plaque formation, and epithelial cell signaling in conjunction with the formation of microcolonies of 10-100 diplococci on the epithelial cell surface within 4-6 hours of infection of cultured cervical epithelial cells (Dietrich et al., 2011; Lee et al., 2005; Merz et al., 1999; Merz and So, 1997, 2000; Merz et al., 2000). The strength of PilT retraction may influence microcolony formation and Gc's ability to activate EGFR signaling during infection (Hockenberry et al., 2016). While pili play a role in adherence and stimulation of host cell

signaling, pilin variation does not affect the ability of Gc to replicate in association with epithelial cells or to exit from epithelial cells (Criss and Seifert, 2006).

Opa proteins interact with human carcinoembryonic antigen cell adhesion molecules (CEACAMs) and heparan sulfate proteoglycans (HSPGs) on host cells to mediate intimate attachment and uptake (Chen et al., 1997; Hauck and Meyer, 2003; Kupsch et al., 1993; Sadarangani et al., 2011; van Putten and Paul, 1995; Wang et al., 1998). There are ~11 individual *opa* genes in Gc, and various Opa proteins have different human receptor specificity and confer different phenotypes in host cell interaction studies (Bhat et al., 1991; Connell et al., 1990; Dempsey et al., 1991; Merz and So, 2000; Obergfell and Seifert, 2015). Opa proteins undergo ON/OFF phase variation mediated by slipped-strand mispairing of pentameric tandem repeats in multiple genes encoding Opa proteins (Murphy et al., 1989; Obergfell and Seifert, 2015; Stern et al., 1986). During human infection, Opa-expressing Gc are recovered from male urethral exudates, from the female cervix during certain points in the menstrual cycle, and from the lower genital tract of experimentally infected female mice, suggesting a role for Opa during infection (Cole et al., 2010; James and Swanson, 1978; Jerse, 1999; Johnson and Criss, 2011).

In the cervix, CEACAMs localize to the apical side of the epithelium while HSPGs are located basolaterally (Mertens et al., 1996; Tchoupa et al., 2014). Opa-CEACAM interactions at the apical epithelial cell surface mediate attachment and internalization to epithelial cells expressing CEACAM1, -5, and -6 using an internalization pathway distinct from the phagocytic pathway seen in neutrophils mediated by CEACAM3 (Billker et al., 2002; Tchoupa et al., 2014; Wang et al., 1998). This process is dependent on CEACAM clustering in membrane domains, as CEACAM-dependent bacterial uptake in epithelial cells is inhibited by cholesterol depletion (Tchoupa et al., 2014). Importantly, while many cancer-derived and immortalized epithelial cell lines do not express CEACAM, Fichorova

et al. detected CEACAM expression in End1 E6/E7 cells used in this work using a pan-CEACAM antibody (recognizing CEACAM1, -5, -6, and -8) (Fichorova et al., 2001).

In primary cervical epithelial cell models, epithelial production of alternative pathway complement components and their deposition on Gc leads to CR3-dependent invasion involving porin and the type IV pilus binding the I-domain of CR3 (Edwards et al., 2002). Opa proteins are dispensable for CR3-mediated adherence and invasion in this model (Edwards and Apicella, 2004). Additionally, Gc releases a protein with phospholipase D (PLD) activity, which has been shown to enter primary cervical epithelial cells and interact with host signaling molecules to increase CR3 on the cell surface and mediate Gc interactions with the epithelial membrane (Edwards et al., 2003). While CR3 has been shown to play a role in adherence and invasion of primary cervical epithelial cells, immortalized cell lines such as the End1 E6/E7 cells used in this work have not been shown to express CR3 (Edwards et al., 2001). Importantly, Gc can adhere to and invade immortalized human epithelial cell lines, indicating that multiple mechanisms can mediate these processes (Merz and So, 2000).

While type IV pili and Opa proteins contribute to host cell adherence and downstream signaling, non-piliated and/or Opa-deficient Gc can also attach to and interact with epithelial cells via other bacterial surface features including LOS and/or porin (Dehio et al., 2000; Merz and So, 2000; Song et al., 2000). Of note, while gonococcal porin is immunogenic, it is closely associated with LOS (Hitchcock, 1984) and reduction modifiable protein (Rmp, aka P.III) (Blake et al., 1989) on the bacterial surface, impeding development of a productive antibody response or vaccine against porin (Edwards and Apicella, 2004). Another unique feature of gonococcal porin is that it is able to translocate into host cell membranes to form a voltage-gated channel (Rudel et al., 1996). Interestingly, this activity of porin has been shown to contribute to epithelial cell apoptosis in cell models of FRT while preventing apoptosis in male urethral epithelial

cell models (Binnicker et al., 2003; Dehio et al., 2000; Massari et al., 2000; Muller et al., 2000; Muller et al., 1999). Further, porin may influence neutrophil activity, with some reports indicating that Gc porin can inhibit degranulation (Lorenzen et al., 2000).

Gc LOS also influences bacterial-bacterial and bacterial-epithelial interactions (Blake et al., 1995). Gc surface feature variation is important for survival in the human host and is also important during transmission. LOS oligosaccharides can vary based on substrate and enzyme availability, influencing host cell interactions and tissue tropism (Burch et al., 1997; Edwards and Apicella, 2004; Yang and Gotschlich, 1996). Additionally, LOS can be sialylated on its lipid A moiety, conferring resistance to antibodies and complement in human serum (van Putten, 1993), but must be desialylated by sialidases of the cervico-vaginal microbiome before being able to infect male urethral cells upon transmission (Harvey et al., 2001; Ketterer et al., 2016). In addition to the surface structures discussed here, Gc expresses many surface lipoproteins important for immune evasion, nutrient acquisition, and regulation of the bacterial stress response in the human host. The reader is referred to a recent comprehensive review of Gc surface lipoproteins (SLPs) and the Slam outer membrane protein implicated in SLP biogenesis (Hooda et al., 2017).

1.3 Gc stimulates a non-protective neutrophilic inflammatory response in the female reproductive tract

The initial site of Gc infection in women is the endocervix, the transition from the lower to upper FRT (Figure 2). Despite neutrophilic cervicitis, the majority of women with cervical gonorrhea do not report symptoms (Edwards and Butler, 2011; Jerse and Deal, 2013). Although genital mucosal secretions contain CAMPs and other bactericidal components, Gc survives in their midst (Figure 1). The MisR-MisS two-component

regulatory system confers inducible resistance in Gc to CAMPs by directing the expression of genes important for envelope integrity (Kandler et al., 2016), which enhances Gc colonization and extends infection duration in a mouse model of lower FRT gonorrhea (Gangaiah et al., 2017).

The recruitment and activation of neutrophils in response to Gc is coordinated by cellular and soluble factors. In the FRT, Gc interacts with epithelial and immune cells, including macrophages, dendritic cells, T cells, and neutrophils, to elicit the local production of inflammatory mediators and activation of a Th17-type response (Figure 3) (Criss and Seifert, 2012; Liu et al., 2011; Masson et al., 2014; Masson et al., 2015). Moreover, Gc infection was recently shown to activate non-muscle myosin II in human cervical tissue, leading to epithelial junctional disruption, exfoliation of endocervical cells, and bacterial subepithelial penetration (Wang et al., 2017).

1.3.1 *Pattern-recognition receptor activation by gonococcal pathogen-associated molecular patterns*

During infection, cytokine production is driven by signaling via epithelial and resident immune cell PRRs that recognize Gc PAMPs (Figure 3). Levels of PRRs, particularly TLR4, which recognizes the lipid A moiety of Gc LOS, and numbers of myeloid cells both increase from the lower to upper FRT, such that an immune response is mounted only to pathogens that ascend into the upper FRT and not to the resident microbiota of the lower FRT (Figure 2). In fact, antimicrobial defenses of FRT cells from various areas of the FRT have been shown to be active against invading pathogens while sparing resident microbiota, like lactobacilli, which are important for the health of the vaginal microbiome (Wira et al., 2011). In response to *N. gonorrhoeae,* epithelial and resident myeloid cells secrete proinflammatory cytokines, including TNF-α, IL-1β, IL-6,

and IL-8 and sentinel Th17 cells release IL-17 (Criss and Seifert, 2012; Dietrich et al., 2011; Feinen et al., 2010; Fichorova et al., 2001; Harvey et al., 2002; Masson et al., 2015; Muenzner et al., 2002; Naumann et al., 1998; Naumann et al., 1997). These cytokines establish a gradient that serves to recruit neutrophils from the bloodstream and activate them at the site of infection.

PRRs include membrane-associated Toll-like receptors (TLRs) and cytoplasmic NOD-like receptors (NLRs) that recognize a variety of Gc PAMPs (Figures 2 & 3) (Chan and Dillard, 2017; Magalhaes et al., 2011; McClure and Massari, 2014). Cervical epithelial cells respond to *N. gonorrhoeae* through stimulation of TLR2 but not TLR4, whereas resident immune and upper FRT cells, including Fallopian tube epithelial cells, are particularly poised to respond to LOS via TLR4 (Fazeli et al., 2005; Fichorova et al., 2002). Gonococcal porin and other lipoproteins stimulate TLR2 to activate NF-kB-driven inflammatory cytokine production (Fisette et al., 2003; Massari et al., 2002). Similarly, TLR4 is potently activated by the lipid A portion of lipooligosaccharide from *N. gonorrhoeae* and its close relative, *Neisseria meningitidis* (John et al., 2017). PG is primarily recognized by intracellular NLRs (Chan and Dillard, 2017; Mavrogiorgos et al., 2014). Recent work from Gray-Owen and colleagues has identified a new PAMP from Gc, the LOS biosynthesis pathway intermediate heptose-1,7-bisphosphate (HBP) (Gaudet and Gray-Owen, 2016; Gaudet et al., 2017; Gaudet et al., 2015). Activation of NF-kB signaling by HBP occurs via phosphorylation-dependent oligomerization of TRAF-interacting protein with forkhead-associated domain (TIFA) (Gaudet et al., 2015). Recognition of HBP represents a novel mechanism to detect and mount an immune response to Gram-negative bacterial pathogens (Gaudet et al., 2017); this may be particularly important for signaling in sentinel cells that lack TLR4 expression, such as cervical epithelial cells.

1.3.2 *Gc modulates innate immune cell responses*

There is strong evidence to suggest that Gc skews innate immune cell recognition and response as an element of its survival strategy. Addition of phosphoethanolamine (PEA) to lipid A by the enzyme LptA, which is found in the pathogenic *Neisseria*, not only enhances TLR4 recognition to stimulate NF-kB-driven cytokine production, but also aids the bacteria in defense against CAMPs found in mucosal secretions and neutrophils (Handing and Criss, 2015; Hobbs et al., 2013; Packiam et al., 2014). Therefore, the potentially damaging effects of lipid A-mediated activation of TLR4 and subsequent inflammation are mitigated by the intrinsic defense against killing that is conferred by modifying lipid A with PEA. Another example was recently elucidated by Golenbock and colleagues, who showed that exposure to Gc leads to STING-dependent activation of the intracellular DNA sensor cyclic GMP-AMP synthase (cGAS) in monocytes and macrophages (Andrade et al., 2016). In combination with TLR4 activation, this stimulates the production of IFN-β. Rather than helping to control infection, IFN-β increases the availability of intracellular iron in macrophages and neutrophils, which is correlated with enhanced survival of Gc (Andrade et al., 2016). Gc may have additional mechanisms to survive and replicate in association with macrophages (Chateau and Seifert, 2016), including through the expression of surface-exposed factors such as macrophage infectivity potentiator-like protein (MIP) (Leuzzi et al., 2005; Reimer et al., 2016).

Not all features of Gc are immunostimulatory. Gc has been reported to polarize macrophages to an immune-regulatory, M2 phenotype, which would downregulate the antimicrobial activity and proinflammatory cytokine production of macrophages (Ortiz et al., 2015). Moreover, while the peptidoglycan (PG) fragments released by Gc are toxic to Fallopian tube cells, they are poorly recognized by innate immune receptors.

Specifically, Duncan and Dillard and colleagues recently found that the PG monomers released by Gc, via the activities of the lytic transglycosylases LtgA and LtgD, are poor activators of mouse NOD2 and TLR2, compared with multimeric PG. This is due to the anhydro moiety on the terminus of Ltg-cleaved fragments, rather than a free reducing (hydroxyl) end, as would be found following digestion by lysozyme (Knilans et al., 2017). These studies suggest that the large amounts of PG fragments released by Gc during normal growth may serve as decoys, to limit PRR activation in the lower FRT and enhance overall Gc survival.

1.3.3 *Gc manipulates the adaptive immune response, skewing the inflammatory environment to attract and activate neutrophils*

Despite the proinflammatory nature of Gc PAMPs, the subsequent host immune response is not sufficient to clear infection. Gc has a remarkable ability to evade host antibody-mediated immunity, due to extensive antigenic and phase variation of its immunogenic surface structures as well as by expression of Rmp, which limits the generation of bactericidal antibodies (Obergfell and Seifert, 2015; Zhu et al., 2011). Gc also manipulates cellular immune responses to limit adaptive immune cell activation and direct the immune response towards a neutrophilic, nonprotective presentation, as described below.

1.3.3.1 *CEACAM1-mediated limitation of immune cell activation*

Most members of the family of Opa outer-membrane proteins of Gc interact with human CEACAM1, which is expressed on T cells, B cells, dendritic cells, and epithelial cells. CEACAM1 has an immunoreceptor tyrosine-based inhibition motif (ITIM) on its

cytoplasmic tail, which recruits the SHP phosphatase to block signaling *in trans* from activating receptors (Gray-Owen and Blumberg, 2006). Engagement of CEACAM1 by Opa+ Gc also drives bacterial internalization into epithelial cells, within which Gc can survive while avoiding exposure to CAMPs, antibodies, complement factors, and other bactericidal components. The importance of the Opa-CEACAM1 interaction to the pathogenesis of gonorrhea is reflected in the strong selection for CEACAM1-binding Gc *in vivo* (Sintsova et al., 2015). However, the *opa* genes phase-vary at high frequency and diversify by recombination and mutation, changing their ability to engage CEACAM1 (Wachter and Hill, 2016). While a role for Gc-CEACAM1 interaction has been described for T cells, B cells, dendritic cells, and epithelial cells, a role for Gc interaction with CEACAM1 on neutrophils has not yet been fully elucidated.

1.3.3.2 *TH17 response*

Gc infection induces the local production of TGF-β and IL-10 to skew the resulting adaptive response away from Th1 and Th2-driven immunity and towards a Th17 response, which in the context of gonorrhea enhances inflammation and prevents the development of protective immunity (Jerse and Deal, 2013; Liu et al., 2012; Liu et al., 2014; Liu and Russell, 2011). Th17 cells produce IL-17, which is important for neutrophil recruitment in the human and mouse lower FRT in response to Gc (Feinen et al., 2010; Masson et al., 2015). The Russell group has proposed reorientation of the T cell response to Gc away from Th17 and towards Th1 as a therapeutic intervention to ameliorate neutrophilic inflammation. In support of this possibility, female mice immunized with Gc antigens in combination with IL-12, a Th1-activating cytokine, clear Gc more rapidly when first infected and are protected against reinfection (Liu et al., 2017).

1.4 Gc thwarts neutrophil functions to enhance its survival and promote continued inflammation

The predominant clinical feature of acute gonorrhea is the presence of viable Gc in association with neutrophils in mucosal secretions (Figure 1). While neutrophils have a robust antimicrobial arsenal (Figure 4), our laboratory and others have identified mechanisms Gc uses to evade killing by neutrophils (Figure 4 & Table 1).

1.4.1 *Gc evades a robust neutrophil antimicrobial arsenal*

Neutrophil antimicrobials include cationic antimicrobial peptides (CAMPs), proteases, proteins that sequester essential metals, and reactive oxygen species (ROS) (Figure 4). These components are released into microbe-containing phagosomes, extracellularly by degranulation, and in chromatin-based neutrophil extracellular traps (NETs) (Figure 4). Despite robust recruitment of neutrophils to the site of Gc infection, viable Gc can still be cultured from gonorrheal secretions suggesting that at least a subset of Gc evade neutrophil killing (Wiesner and Thompson, 1980). Our laboratory and others have uncovered two ways used by Gc to survive exposure to neutrophils (see (Criss and Seifert, 2012; Johnson and Criss, 2011) for overviews): Gc possesses defenses against neutrophil antimicrobial components and also limits neutrophils from producing and releasing them (Table 1).

Neutrophils combat invading pathogens using both oxidative and non-oxidative killing mechanisms that can act extracellularly, through release of granule components or NETs, or intracellularly, through release into phagolysosomes. However Gc has evolved a number of mechanisms to evade neutrophil killing, reviewed in (Johnson and

Criss, 2011) and (Criss and Seifert, 2012), discussed here, and summarized in Table 1. Gc encodes a superoxide dismutase, a catalase, peroxidases, and a methionine sulfoxide reductase, RecN, to defend against and repair damage induced by ROS (Seib et al., 2005; Seib et al., 2004; Soler-Garcia and Jerse, 2004; Stohl et al., 2005; Stohl and Seifert, 2006; Wilks et al., 1998). RecN may also help Gc evade neutrophil killing by other mechanisms (Stohl et al., 2005). Additionally, PorB, the essential Gc porin has been shown to inhibit the neutrophil oxidative burst and generation of ROS (Chen and Seifert, 2011; Lorenzen et al., 2000). Gc type IV pili have been shown to protect Gc against neutrophil CAMPs and ROS (Stohl et al., 2013). Gc also encodes a PG peptidase, Mpg that contributes to type IV pili-mediated resistance to CAMPs and ROS (Stohl et al., 2012; Stohl et al., 2005; Stohl et al., 2013). Gc lactate dehydrogenases LdhA and LdhD may help Gc use lactate as a carbon source and contribute to survival in neutrophils (Atack et al., 2014).

Nutritional immunity, the sequestration of essential nutrients including Zn, is an important host defense against pathogens. Recent work demonstrated that Gc uses TonB-dependent transporters to overcome sequestration of Zn by directly binding calprotectin, a host Zn-binding protein that is produced by neutrophils and contained in NETs. Expression of TdfH affords Gc a survival advantage when in association with NETs, in a Zn-dependent manner (Jean et al., 2016). We also recently described a mechanism by which Gc uses a nuclease to degrade NET DNA (Juneau et al., 2015). Importantly, Gc induces NET formation but efficiently evades NET killing, which may contribute to the sustained and inefficient neutrophil response to infection (Juneau et al., 2015).

LOS modifications have also been shown to contribute to Gc resistance to neutrophil killing mechanisms. Gc Lst inhibits phagocytosis by neutrophils in suspension by sialylating N-lactotetraose-containing LOS (Kim et al., 1992; Rest and Frangipane,

1992). More recently, phosphoethanolamine addition to gonococcal LOS by LptA was demonstrated to help Gc overcome neutrophil killing mechanisms by limiting phagolysosome maturation as well as helping Gc resist antimicrobial components of neutrophil granules delivered to the phagosome, released extracellularly, and contained in NETs (Handing and Criss, 2015). Gonococcal LtgA and LtgD are important for peptidoglycan release during Gc growth. A recent study from our lab showed that these enzymes also help Gc resist killing by neutrophils, in particular by resisting killing by lysozyme (Ragland et al., 2017). Importantly, this study also demonstrated that LtgA and LtgD activity reduces neutrophil activation by reducing granule mobilization to the phagosomal and plasma membranes (Ragland et al., 2017). An example of a defense mechanism shared with other pathogens includes Gc MIP, a peptidyl-prolyl cis/trans isomerase activity found in *Chlamydia* and pathogenic *Neisseria*. Using two newly identified inhibitors of MIP, PipN3 and PipN4, recent findings support a role for MIP during gonococcal survival in neutrophils by a yet uncharacterized mechanism (Reimer et al., 2016). These examples of mechanisms Gc uses to evade neutrophil antimicrobial activity provide strong evidence for the relevance of neutrophil-evasion in the context of infection. Further, they support the hypothesis that Gc has evolved to stimulate and then effectively evade the non-protective neutrophil response in order to persist and be transmitted.

1.4.2 *Neutrophil antimicrobial activity that is insufficient to clear Gc can contribute to host cell damage and enhance neutrophilic inflammation*

Antimicrobial components are a crucial part of neutrophils' functionality, but they have the potential to damage host cells. ROS oxidize lipids, proteins, and DNA, and also serve as a second messenger to enhance inflammation and neutrophil recruitment

(Mittal et al., 2014). Proteases, including neutrophil-derived matrix metalloproteases (MMPs), degrade tissue extracellular matrix and contribute to sustained neutrophil recruitment (Kruger et al., 2015). The CAMP LL-37 interacts with host cell receptors including TLR4 and contributes to their continued activation during infection and inflammation (Verjans et al., 2016). Histones, released from neutrophils in NETs, induce direct epithelial cell damage (Saffarzadeh et al., 2012). NETs are also thought to contribute to the pathology associated with autoimmune diseases by providing self-antigen (such as in Systemic Lupus Erythematosus), and with cardiovascular disease by enhancing formation of atherosclerotic plaques (Doring et al., 2017; Papayannopoulos and Zychlinsky, 2009). NETs are formed in response to Gc, but the bacteria have multiple mechanisms to survive in association with NETs, including resistance to antimicrobials in NETs, acquisition of calprotectin by TdfH, maintenance of envelope integrity, and expression of Nuc that degrades NET DNA (Figure 4 & Table 1) (Gunderson and Seifert, 2015; Handing and Criss, 2015; Jean et al., 2016; Juneau et al., 2015). Although the contribution of Gc-induced NETs to cellular damage remains to be elucidated, it is likely that Nuc could release CAMPs and histones from NETs, which would directly damage epithelial cells and stimulate continued inflammation. Thus neutrophils' antimicrobial components have the capacity to directly damage host cells as well as promote continued neutrophilic inflammation.

1.4.3 *Gc defenses against neutrophils also modulate neutrophil activation*

Some of the defenses used by Gc against neutrophil antimicrobial activities influence neutrophil activation and extracellular release of antimicrobial components (Figure 4). These components may contribute to the cellular damage associated with gonorrhea in the FRT.

Variations in Opa protein expression contribute to neutrophil activation and the ability of Gc to survive exposure to neutrophils. While Opa-CEACAM1 interactions with T cells, B cells, and dendritic cells may contribute to immunosuppression during infection as discussed in section 1.3.3.1, the role for this interaction during interaction with neutrophils is poorly characterized. In comparison, a subset of Gc expresses Opa proteins that interact with CEACAM3, which unlike CEACAM1, has an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic tail. Interaction with CEACAM3 on neutrophils leads to increased ROS production and granule release, and decreased Gc survival (Figure 4) (Johnson et al., 2015; Sarantis and Gray-Owen, 2007, 2012; Sintsova et al., 2014; Smirnov et al., 2014). Engagement of CEACAM3 also serves to further recruit and activate neutrophils at the site of infection *in vivo* (Sintsova et al., 2014). The same study that found a selection for an Opa protein repertoire that binds CEACAM1 also found a selection against CEACAM3-binding Opa proteins in patient isolates, underscoring the importance for Gc of avoiding recognition by this receptor (Sintsova et al., 2015).

Some Gc defenses also limit the degree of granule fusion with phagosomes and the plasma membrane. We recently reported that addition of PEA to LOS by LptA is important for survival of Gc from neutrophils, not only by defending against CAMPs, but also by limiting the extent of phagolysosome formation (Handing and Criss, 2015). This was surprising since PEA-modified lipid A has a higher affinity for TLR4 (Packiam et al., 2014), suggesting the activation of neutrophils by Gc may be TLR4-independent. As another example, we found the PG modifying enzymes LtgA and LtgD defend Gc from neutrophils, particularly from killing by lysozyme, and limit granule fusion with phagosomes and the plasma membrane (Ragland et al., 2017). This observation is in agreement with recent findings that PG fragments produced by LtgA and LtgD are nonstimulatory for NLRs and TLRs (Knilans et al., 2017).

1.4.4 *Challenges in modeling ascending infection with Gc and the consequences of neutrophil influx*

A direct role for neutrophils in inducing epithelial cell damage in gonorrhea has not yet been described owing to challenges of studying Gc infection and neutrophilic inflammation in the FRT. *Ex vivo* systems such as cell lines and tissue explants do not generally incorporate neutrophils, although basal-to-apical transepithelial migration in response to other mucosal pathogens has been modeled with monolayers of polarized epithelial cells (Szabady and McCormick, 2013). A mouse model of lower FRT infection has been established by the Jerse group and used to probe the bacterial and host factors that are important for colonization and early neutrophil recruitment (Jerse et al., 2011). Subsequently, Gc infection models using mice transgenic for human receptors and other human-specific components have been developed. In particular, in mice that are transgenic for human CEACAMs, the Gray-Owen group has reported that Gc robustly infects the lower FRT and triggers a strong influx of CEACAM-expressing neutrophils that interact with the bacteria (Sintsova et al., 2014). While mouse models have increased our understanding of Gc colonization and early inflammatory responses, at this time they do not reproduce features of Gc infection seen in women, including ascending infection, sustained neutrophil influx, and epithelial damage.

1.5 Neutrophilic inflammation and epithelial damage in response to Gc in the upper female reproductive tract

If treatment of Gc infection does not occur or is ineffective, Gc can ascend to the upper FRT, where the neutrophilic inflammatory response is more potent; this is

correlated with increased epithelial expression of pattern recognition receptors (PRRs) and greater numbers of resident myeloid cells that are poised to detect foreign antigens (Figure 2) (Wira et al., 2005). The interactions of Gc with epithelial cells during ascent from the lower to the upper FRT have recently been simulated using human Hec-1-A cells cultured in a bioreactor as three-dimensional organoids (Laniewski et al., 2017). Gc infection, but not colonization with commensal *Lactobacillus crispatus* or *Gardnerella vaginalis*, stimulates production of proinflammatory mediators (IL-1β, IL-8, and TNF-α) and alterations to host cells, including microvillus remodeling (Laniewski et al., 2017).

A hallmark of upper FRT infection with Gc is the death of ciliated cells lining the Fallopian tube. Reduced motility in the Fallopian tube, along with tubal scarring, result in infertility and ectopic pregnancy. Human Fallopian tube explants have been instrumental in demonstrating that release of LOS and PG fragments by Gc stimulate the production of inflammatory cytokines and second messengers, including TNF-α and nitric oxide (Gregg et al., 1981; Melly et al., 1981; Melly et al., 1984). Unlike most bacteria, including other *Neisseria* species, Gc poorly recycles its PG during cell wall turnover (Woodhams et al., 2013). Instead, PG fragments are released extracellularly and are responsible for ciliated cell death (Chan and Dillard, 2016, 2017). A new contributor to Fallopian tube damage was recently reported by Velazquez, Christodoulides, and colleagues (Rodas et al., 2016). Gc-infected Fallopian tube epithelial cells increase production of MMPs and extracellular release of MMP-9, which may amplify tissue destruction by degrading extracellular matrix and interfering with tissue repair. Further, MMP-9 generates chemokine mimetics and extracellular matrix fragments, which further stimulate inflammation and neutrophil recruitment. Neutrophils themselves are a significant source of MMP-9, setting up a vicious cycle of inflammation and epithelial cell damage (Kruger et al., 2015).

Much remains to be learned about the mechanisms underlying Gc-induced neutrophilic inflammation and damage in the upper FRT. It is unethical to conduct human challenge studies on women due to the risk of serious complications. Fallopian tube explants and endometrial organoids are useful models, especially if primary human neutrophils are introduced. A new female mouse model of upper FRT infection with Gc provides an *in vivo* platform for studying neutrophilic inflammation and its consequences (Islam et al., 2016). In this model, transcervical inoculation of Gc leads to infection of the uterine horns and corpus of the upper FRT, resulting in robust and rapid neutrophilic infiltration, edema, and other signs of PID (Islam et al., 2016). The inflammatory process is exaggerated in mice in diestrus; similarly, PID most commonly presents after the onset of menses in women with gonorrhea (Islam et al., 2016). Introduction of transgenic or knockout mice into this model may enable longer-term infection with Gc, to model the chronic inflammation and consequent damage associated with PID.

1.6 Modeling neutrophil transepithelial migration *in vitro*

Circulating neutrophils are quiescent, becoming activated only when recruited from the bloodstream to sites of inflammation (Borregaard, 2010). Chemotactic gradients generated in response to infection promote neutrophil transendothelial migration out of blood vessels and through the interstitium to the site of infection (Borregaard, 2010; Szabady and McCormick, 2013). Endothelial cells lining postcapillary beds are stimulated by inflammatory biomarkers such as IL-17 to express selectins and integrins that bind ligands on neutrophils (Borregaard, 2010; Szabady and McCormick, 2013). Neutrophils then leave the bloodstream via sequential rolling, firm adhesion, and transendothelial migration (Borregaard, 2010; Szabady and McCormick, 2013). During transendothelial migration, neutrophils migrate via a paracellular, between cells, or

transcellular, through cells, route (Borregaard, 2010; Muller, 2003). As neutrophils move paracellularly, they reversibly remodel epithelial tight junctions (Nash et al., 1987, 1988). Chemotactic gradients of inflammatory biomarkers, including IL-8, have then been shown to recruit neutrophils through the extracellular matrix to sites of infection and inflammation, but not to drive transepithelial migration or neutrophil-driven epithelial cell damage (Borregaard, 2010; Eckmann et al., 1993; Kucharzik et al., 2005; McCormick et al., 1995; Szabady and McCormick, 2013). In the tissue, neutrophils become poised to deploy a robust antimicrobial arsenal (Borregaard, 2010; Condliffe et al., 1998; Szabady and McCormick, 2013). Once they have reached the site of infection, neutrophils must complete a final step of transmigration across the epithelium and into the lumen. The signals driving transepithelial migration to mucosal bacterial pathogens have recently been elucidated using *in vitro* models of intestinal and pulmonary mucosal epithelia (Bhowmick et al., 2013; Boll and McCormick, 2012; Boll et al., 2012a; Boll et al., 2012b; Hurley et al., 2004; Kohler et al., 2002; McCormick et al., 1998; Mrsny et al., 2004; Pazos et al., 2017; Pazos et al., 2015; Tamang et al., 2012; Zurawski et al., 2006), but remain unexplored in the context Gc infection of the cervix and FRT.

1.6.1 *Mechanisms of neutrophil transepithelial migration*

Once neutrophils are recruited from the bloodstream by chemoattractants such as IL-8, they follow additional chemotactic gradients to the site of infection. In fact, studies suggest that neutrophils preferentially follow other chemoattractants through the extracellular matrix including eicosanoids like leukotriene B_4 (LTB_4), as well as neutrophil chemoattractants that might be uniquely generated at sights of bacterial infection including the complement fragment C5a and bacterial-derived formyl peptides (Foxman et al., 1997; Heit et al., 2002; Szabady and McCormick, 2013). These chemoattractants

stimulate G-protein-coupled-receptors (GPCRs) on neutrophils, leading to a calcium flux and downstream signaling to stimulate directed migration (Viola and Luster, 2008). Additional signals are then required to drive neutrophils across epithelial barriers to the lumen at the site of mucosal bacterial infection (Szabady and McCormick, 2013). Recently, the final step of neutrophil transepithelial migration has been shown to be driven by the production of eicosanoids, which will be discussed in further detail in section 1.7 (Bhowmick et al., 2013; Boll and McCormick, 2012; Boll et al., 2012a; Boll et al., 2012b; Hurley et al., 2004; Kohler et al., 2002; McCormick et al., 1998; Mrsny et al., 2004; Pazos et al., 2017; Pazos et al., 2015; Tamang et al., 2012; Zurawski et al., 2006). As during transendothelial migration, neutrophils can migrate across epithelial monolayers either paracellularly across tight junctions or by a transcellular route, with the paracellular route seemingly most common (Szabady and McCormick, 2013). As neutrophils move paracellularly, they reversibly disrupt tight junctions, in part due to the actions of neutrophil proteases (Ginzberg et al., 2001; Nash et al., 1987). Paracellular neutrophil migration requires the integrins CD11b/CD18 (Parkos et al., 1991), similar to during transendothelial migration (Borregaard, 2010). In the setting of transepithelial migration, additional interactions with CD47 and Sirp-α also contribute to neutrophil movement between epithelial cells (Liu et al., 2002; Parkos et al., 1996). Upon reaching the luminal surface, neutrophils first adhere to the apical surface of mucosal epithelial cells and then detach (Brazil et al., 2010; Lawrence et al., 2003).

1.6.2 *Neutrophil transepithelial migration can disrupt epithelial barrier function and lead to mucosal epithelial damage*

Neutrophil transepithelial migration further activates neutrophils against mucosal pathogens (Nadeau et al., 2002; Szabady and McCormick, 2013). Neutrophil activity

may help to clear pathogens, but can also have significant off-target damaging effects on host cells. This is particularly relevant in the setting of gonococcal infection, where neutrophils are not thought to be sufficient at clearing Gc but are recruited in large numbers throughout infection. Notably, large populations of transmigrating neutrophils disrupt epithelial monolayer integrity and barrier function (Nash et al., 1987; Nusrat et al., 1997) and can contribute to pathology associated with neutrophilic inflammation. Given that neutrophils are highly activated following transepithelial migration into the lumen and that neutrophil antimicrobial activity can have off-target effects on surrounding host epithelium, neutrophils can then continue to contribute to the pathology associated with mucosal inflammation (Chin and Parkos, 2007; Fournier and Parkos, 2012; Nash et al., 1987; Nusrat et al., 1997; Szabady and McCormick, 2013).

Epithelial cell damage resulting from neutrophil influx has been extensively studied in the context of gastrointestinal pathology, including in the context of inflammatory bowel diseases (IBDs), ulcerative colitis (UC) and Crohn's disease (CD), in which there is significant neutrophil-rich pathology (Fournier and Parkos, 2012; Maloy and Powrie, 2011). In these contexts, epithelial damage includes epithelial cell death and sloughing, effacement of microvilli, and formation of neutrophil rich crypt abscesses, areas filled with neutrophils in which epithelial cells are either lost or atypical (Fournier and Parkos, 2012). Neutrophil-influx-induced epithelial damage has also been characterized in the lung, for example in the context of acute respiratory distress syndrome (ARDS) where primed neutrophils are retained in the lung and mediate severe epithelial cell damage (Kruger et al., 2015; Summers et al., 2014). It is understood that both *in vitro* and *in vivo*, neutrophils first undergo priming, via interaction with pro-inflammatory signals including TNFα, IL-1β, IL-8, leukotriene B_4 (LTB_4) and GM-CSF as well as bacterial products including LPS, that is then followed by a second stimulus that fully activates neutrophils (Condliffe et al., 1998). This process is important for neutrophil

activity, and contributes to clearance of invading pathogens. However, there is an important balance between neutrophil inflammation and resolution that, when dysregulated as in IBD and ARDS, leads to significant pathology.

1.6.3 *Pro-inflammatory and pro-resolving factors normally help maintain homeostasis and are both important in protection of tissues*

In homeostasis, neutrophil recruitment and activation is followed by resolution. In response to insults including invading microbes and epithelial barrier breach, pro-inflammatory cytokines and chemokines work in concert with pro-inflammatory lipid mediators, including eicosanoids produced from arachidonic acid, to promote neutrophil recruitment (Serhan, 2014). Pro-inflammatory lipid mediators include eicosanoids (prostaglandins, hepoxilins, and leukotrienes) (Figure 5). In particular, prostaglandins and leukotrienes stimulate neutrophil migration from the vasculature to the tissue. Recently, the role for eicosanoids in stimulating neutrophil recruitment and transepithelial migration to mucosal pathogens has been appreciated and will be discussed in detail in section 1.7 of this dissertation. Active resolution of inflammation quickly follows. Pro-resolving lipid mediators include lipoxins, resolvins, and protectins, and are important during resolution of neutrophilic inflammation and return to homeostasis (Figure 5) (Serhan, 2014). If resolution is not successful, dysregulated lipid-mediated inflammation can lead to chronic inflammation, tissue damage, and fibrosis (Serhan, 2014).

1.6.4 *In vitro systems to model and measure neutrophil transepithelial migration*

Our understanding of neutrophil transepithelial migration comes from observations made using *in vitro* models of epithelial monolayers. An early model used

to study this process used T84 cells, a human intestinal epithelial cell line derived from a colon cancer metastasis. Parkos et al. developed a method to culture T84 cells on inverted inserts in order to model physiologically relevant basal-to-apical neutrophil transepithelial migration (Parkos et al., 1991). Since then, a number of cell lines have been used to model apical infection and subsequent neutrophil transepithelial migration to different mucosal pathogens. In the work presented here, we chose immortalized endocervical, End1 E6/E7 (ATCC CRL-2615), cells generated by Fichorova et al. (Fichorova et al., 1997). End1 cells were derived from a premenopausal woman undergoing hysterectomy for endometriosis and immortalized using HPV proteins E6 and E7 (Fichorova et al., 1997). End1 cells maintain stable expression of epithelial differentiation markers and upregulate IL-8, IL-6, intracellular adhesion molecule 1 (ICAM-1 aka CD54) and CD66c when exposed to Gc (Fichorova et al., 2002; Fichorova et al., 2001). Further, polarized End1 cells on inverted inserts support neutrophil transepithelial migration (Fichorova et al., 2005). Given that models to date do not fully recapitulate cervical infection and resultant neutrophil influx in female gonococcal infection, polarized monolayers of End1 cells will provide a physiologically relevant model with which to study this process.

1.7 Bioactive lipids are potent neutrophil chemoattractants

1.7.1 *Eicosanoids and their role in inflammation*

Eicosanoids include prostaglandins, leukotrienes, and hepoxilins (Figure 5). These bioactive lipids are derived from arachidonic acid and other polyunsaturated fatty acids (PUFAs) via the action of lipoxygenase (LOX), cyclooxygenase (COX), or cytochrome P450 enzymes (Dennis and Norris, 2015; Harizi et al., 2008) (Figure 6A).

Eicosanoids can be pro-inflammatory or exhibit pro-resolving activity, with both activities important during homeostasis (Figure 5). For example, eicosanoids and the enzymes that generate them contribute to homeostatic functions including maintaining barrier function and mucosal integrity (Dennis and Norris, 2015). During infection and inflammation, engagement of PRRs stimulates phospholipase A_2 (PLA_2) mediated release of arachidonic acid (AA) from host cell membranes (Dennis and Norris, 2015). PLA_2 activity is tightly regulated within host cells, providing an important level of regulation for eicosanoid production (Cathcart, 2009; Sadik and Luster, 2012). Activation of PLA_2 requires phosphorylation, which can be regulated by protein kinase C (PKC), and calcium-dependent translocation of PLA_2 to cell membranes (Cathcart, 2009; Sadik and Luster, 2012). Release of AA is the rate-limiting step in eicosanoid generation and is mediated primarily by three different PLA_2 isoforms in a context-dependent manner (Dennis and Norris, 2015; Murakami and Kudo, 2002). These include cytosolic calcium-dependent PLA_2 ($cPLA_2$), specifically $cPLA_2\alpha$, secretory PLA_2 ($sPLA_2$), and the calcium-independent $iPLA_2$ (Cathcart, 2009; Murakami and Kudo, 2002). These PLA_2 enzymes liberate free fatty acids by hydrolyzing the fatty acyl group from the *sn-2* position of glycophospholipids (Cathcart, 2009). In this regard, $cPLA_2$ is particularly interesting as a potential player during infection because it selectively cleaves *sn-2* on AA to serve as a precursor for AA-derived bioactive lipids (Cathcart, 2009). Notably, production of different eicosanoids is cell-specific, depending on the particular enzymes expressed, and context specific, with varied activity depending on environmental cues (Dennis and Norris, 2015). An additional level of specificity for eicosanoid signaling is provided by cell- and tissue-specific expression of eicosanoid receptors (Dennis and Norris, 2015). Recently, a number of mucosal pathogens have been shown to stimulate eicosanoid-driven neutrophil influx in pathogen- and context-specific ways that likely contribute to the characteristic neutrophil-mediated pathologies during infection.

1.7.2 *Hepoxilin A_3 and Leukotriene B_4 are particularly potent neutrophil chemoattractants*

Hepoxilin A_3 (HXA_3) and leukotriene B_4 (LTB_4) are particularly potent neutrophil chemoattractants implicated in neutrophil influx to mucosal bacterial infections (Figure 5). HXA_3 contains an epoxide ring that is required for neutrophil chemotactic activity (Figure 6B) (Morisseau et al., 2012; Pace-Asciak, 2015). HXA_3 interaction with neutrophils produces a calcium flux that is sensitive to pertussis toxin (Dho et al., 1990; Pace-Asciak, 2015; Reynaud et al., 1995b; Sutherland et al., 2000), suggesting that its receptor is a GPCR, however the receptor for HXA_3 has not yet been defined. Recent work has shown that HXA_3 is produced by mucosal epithelial cells in response to a number of mucosal bacterial pathogens to drive neutrophil transepithelial migration (Szabady and McCormick, 2013), as will be further discussed in section 1.7.4. HXA_3 is extremely labile, and is quickly degraded to an inert trioxilin A_3 (TrA_3) in an acidic environment or by epoxide hydrolases (Morisseau et al., 2012; Pace-Asciak, 2015) (Figure 6B). Therefore, its actions are likely short-lived, supporting the idea that HXA_3 provides a local chemotactic gradient for neutrophils that is tightly regulated by its degradation.

In contrast to the local chemotactic gradient across mucosal epithelia created by HXA_3, LTB_4 has been shown to play a role in amplifying local chemotactic gradients over large distances (Figure 6C). LTB_4 has both a high affinity, BLT_1, and low affinity, BLT_2 receptor (Tager and Luster, 2003; Yokomizo et al., 1997; Yokomizo et al., 2000). Both receptors are GPCRs and engagement stimulates a calcium-flux in the target cell (Figure 7) (Tager and Luster, 2003). BLT_1 is primarily expressed on myeloid cells, while BLT_2 exhibits a wider expression profile (Tager and Luster, 2003). The LTB_4-BLT_1 axis is important for directing neutrophil chemotaxis and augmenting local gradients of

inflammatory mediators to coordinate robust neutrophil influx at sites of inflammation (Afonso et al., 2012; Nemeth and Mocsai, 2016).

There are some important differences between HXA_3 and LTB_4. Notably, while LTB_4 acts to both recruit and activate neutrophils (Le Bel et al., 2014), HXA_3 is purely a chemoattractant and does not activate neutrophils (Mrsny et al., 2004; Szabady and McCormick, 2013). Neutrophil migration and LTB_4 signaling activates neutrophils against invading pathogens (Le Bel et al., 2014; Nadeau et al., 2002). Neutrophils also directly amplify neutrophil activation and migration in a paracrine and autocrine fashion (Nemeth and Mocsai, 2016). An important example of this is through the paracrine action of neutrophil-derived LTB_4. LTB_4 accumulates at sites of inflammation and interacts with BLT_1 receptors leading to further production of LTB_4 and potentiating both neutrophil migration and activation (Figure 7) (Lammermann et al., 2013; Nemeth and Mocsai, 2016). Therefore, in the context of infection and neutrophilic inflammation, these lipids likely function to first initiate and then amplify neutrophil influx and activation. *In vivo*, multiple additional cell types and factors may also influence the inflammatory milieu during Gc infection. For example, as discussed in section 1.3.3.2, recent work has established that Gc skews the immune response away from a protective adaptive immune response and towards a Th17-driven neutrophilic response important in human gonococcal infection (Feinen et al., 2010; Liu et al., 2012; Liu et al., 2014; Liu and Russell, 2011; Masson et al., 2015). LTB_4 has recently been shown to induce Th17 cell migration to a greater extent than Th1 or Th2 cell migration via BLT_1 on Th17 cells (Lee et al., 2015), which may reveal another role for this important chemoattractant in promoting characteristic neutrophilic influx in the context of Gc infection.

1.7.3 *HXA_3 and LTB_4 are generated from arachidonic acid via 12-lipoxygenase or 5-lipoxygenase activity*

Lipoxygenases mediate the conversion of AA to HXA_3 and LTB_4 (Figure 6A). Human and mouse lipoxygenases are not complementary, highlighting species-specific regulation of these enzymes and their functions (Mashima and Okuyama, 2015). HXA_3 is produced via 12-lipoxygenase activity, exhibited by different lipoxygenase enzymes depending on cell type, tissue distribution, and cytokine-mediated expression level (Mashima and Okuyama, 2015). Human enzymes with 12-lipoxygenase activity include 15-LOX, 12-LOX, and 12R-LOX in conjunction with eLOX-3 (Figure 6A) (Mashima and Okuyama, 2015). 12R-LOX is primarily expressed in skin and is unique among the 12-lipoxygenase enzymes in that it requires the downstream activity of eLOX-3 to generate HXA_3 (Epp et al., 2007; Jobard et al., 2002; Krieg et al., 2013; Mashima and Okuyama, 2015; Yu et al., 2003). In homeostasis, 12R-LOX and eLOX-3 preferentially act on linoleic acid (LA) to produce ceramides that form a protective lipid layer called a corneocyte-lipid envelope (CLE) to help prevent transepithelial water loss (TEWL) (Mashima and Okuyama, 2015). However the 12R-LOX product of AA metabolism, 12R-HETE, has been found in psoriasis scales, which are characteristically neutrophil-rich lesions, suggesting that under inflammatory conditions these enzymes may contribute to HXA_3 production and subsequent neutrophil influx (Boeglin et al., 1998; Mashima and Okuyama, 2015).

LTB_4 is primarily produced by myeloid cells (Figure 7). Following a calcium flux, $cPLA_2$ liberates AA, which is converted to LTB_4 via 5-lipoxygenase activity (Figure 7) (Le Bel et al., 2014). The 5-LOX enzyme translocates to the nuclear membrane and is activated by 5-lipoxygenase-activating-protein (FLAP) (Figure 7) (Dixon et al., 1990). The resulting LTA_4 is then converted to LTB_4 via LTA_4-hydrolase (Figure 7) (Samuelsson

and Funk, 1989). While LTB_4 is primarily produced by myeloid cells, there have been reports of its production by other cell types, for example by 5-LOX in bronchial epithelial cells in the context of inflammation (Jame et al., 2007). Regulation of LTB_4 synthesis is in part regulated by expression of 5-LOX and FLAP (Reid et al., 1990; Rouzer et al., 1986; Samuelsson and Funk, 1989), which is thought to be limited to leukocytes, however LTA_4 hydrolase is more widely expressed (Iversen et al., 1994; Radmark et al., 1989; Samuelsson and Funk, 1989). Additionally, there have been reports that LTA_4 can be processed transcellularly to LTB_4 by cells that do not have 5-LOX (Figure 7) (Fabre et al., 2002; Sadik and Luster, 2012). In this process, LTA_4 produced by myeloid cells can be released, delivered into surrounding cells that do not express 5-LOX, and converted to LTB_4 via LTA_4 hydrolase (Figure 7) (Bigby, 1991; Claesson and Haeggstrom, 1988; Fabre et al., 2002; Fitzpatrick et al., 1984; Grimminger et al., 1988; Grimminger et al., 1991; Iversen et al., 1994; McGee and Fitzpatrick, 1986; Sala et al., 1996). Therefore, transcellular production of LTB_4 from LTA_4 during inflammation may contribute to immune cell recruitment.

1.7.4 *Several important mucosal pathogens stimulate eicosanoid production in unique, site-specific ways*

Robust neutrophilic inflammation is associated with a number of mucosal infections with pathogenic bacteria including *Salmonella, Shigella flexneri,* Enteroaggregative *Escherichia coli, Streptococcus pneumoniae,* and *Pseudomonas aeruginosa*. Specifically, these mucosal pathogens elicit epithelial production of HXA_3 to initiate neutrophil transepithelial migration (Bhowmick et al., 2013; Boll and McCormick, 2012; Boll et al., 2012a; Boll et al., 2012b; Hurley et al., 2004; Kohler et al., 2002; Mrsny et al., 2004; Tamang et al., 2012; Zurawski et al., 2006). A recent report has

demonstrated that LTB_4 amplifies neutrophil transepithelial migration to *P. aeruginosa* (Pazos et al., 2015). The specific way in which various bacteria affect these signaling pathways has been shown to be exquisitely pathogen and context-specific, suggesting a pattern where individual bacteria have evolved unique mechanisms to co-opt/exploit eicosanoid-driven neutrophilic inflammation. In all cases, the bacterial pathogens influence the generation of AA, the rate-limiting step in HXA_3 production (Figure 6A). Of the pathogens shown to activate PLA_2, *S. flexneri* and *S. pneumoniae* specifically activate epithelial $cPLA_2\alpha$ required for neutrophil transepithelial migration (Bhowmick et al., 2017; Mumy et al., 2008). In contrast, *P. aeruginosa* also stimulates PLA_2-dependent neutrophil recruitment, but while $cPLA_2\alpha$ is activated in airway epithelial cells in this context, epithelial $cPLA_2\alpha$ is not required for neutrophil transepithelial migration (Hurley et al., 2011). Instead, Hurley et al. found that in response to *P. aeruginosa*, epithelial $cPLA_2\alpha$ led to increased production of PGE_2 (Hurley et al., 2011). Findings from the Hurley group suggest that HXA_3 from infected epithelial cells directs initial neutrophil transmigration that is amplified by neutrophil production of LTB_4 (Pazos et al., 2015; Tamang et al., 2012). *P. aeruginosa* encodes a type III secretion system (T3SS) to inject bacterial factors directly into host cells. One of the T3SS effectors secreted by *P. aeruginosa* is ExoU, which has been linked to virulence of this pathogen (Saliba et al., 2005). Interestingly, ExoU is cytotoxic to host cells *in vitro* in a PLA_2-dependent and lipase-activity-dependent manner (Phillips et al., 2003; Sato et al., 2003). Additionally, ExoU has acyl hydrolase activity and can act on lipids and phospholipids, exhibiting PLA_2-like phospholipase activity (Phillips et al., 2003; Sato et al., 2003). In fact, ExoU increases AA release and eicosanoid production in infected endothelial cells (Saliba et al., 2005) and from neutrophils in a model of neutrophil migration across infected pulmonary epithelial cells (Pazos et al., 2017).

Given that Gc does not encode a T3SS, there is an important difference between Gc and bacterial pathogens known to stimulate eicosanoid-driven neutrophil influx via T3SS effectors, including *S.* Typhimurium and *P. aeruginosa* (Criss et al., 2001; Lee et al., 2000; Pazos et al., 2017; Silva et al., 2004). Gc also differs from other mucosal bacterial pathogens in the surface features it uses to adhere to and interact with host cells. Enteroaggregative *E. coli* (EAEC) stimulates HXA_3 production via adherence factors, with the fimbriae of EAEC stimulating AA release (Boll et al., 2012b). *S. flexneri* employs both T3SS secreted factors and adherence factors to interact with multiple epithelial players involved in generating HXA_3 (Kohler et al., 2002; Zurawski et al., 2006).

Given that Gc elicits a robust neutrophilic response and that eicosanoids play a role in a number of neutrophilic inflammatory processes, we hypothesized that the potent neutrophil chemoattractants HXA_3 and LTB_4 are involved in the context of this important human pathogen. The cervix is a unique epithelial site because it marks a transition between a keratinized epithelium (skin-like) to a single columnar epithelium (mucosal). Thus we also sought to define how Gc might interact with these pathways at this unique transitional site. Importantly, since Gc does not encode a T3SS or the adherence factors other bacteria use to interact with these bioactive lipid pathways, these studies will pave the way for future work to discover Gc factors that modulate the neutrophilic inflammatory response.

1.8 Dissertation goals and significance

Gc induces potent neutrophilic inflammatory responses, yet survives in their midst. Sustained infection and neutrophilic inflammation likely underlie the pathology associated with gonorrhea in women. While current models have been used to study interactions of Gc with host cells, including epithelial and immune cells, we do not

understand how Gc stimulates neutrophil transepithelial migration at the site of infection. This is particularly important in the context of female infection, where our current models do not completely mimic cervical infection and neutrophil migration, and where robust neutrophilic inflammation likely contributes to the severe clinical sequelae associated with female gonococcal infection. Thus, the goal of this dissertation is to address an important unanswered question in the field: what are the signals that coordinate neutrophil transepithelial migration in a relevant model of cervical infection with Gc?

Here I will investigate the mechanism by which endocervical cells and neutrophils coordinate neutrophil transepithelial migration to Gc. Importantly, understanding the signals that drive neutrophil influx may reveal adjunctive therapeutic targets to try to limit deleterious neutrophil influx while administering antibiotics to clear Gc. Recently developed models of infection, including a mouse model of PID (Islam et al., 2016), will provide future platforms for translating the work presented here to studying neutrophil influx, measuring consequent epithelial cell damage, and testing novel therapeutics to thwart the non-productive, sustained neutrophil response to Gc. These advances will enable a better understanding of how sustained neutrophilic inflammation in response to Gc drives epithelial damage and serious clinical sequelae in women.

1.9 Figures for Chapter 1

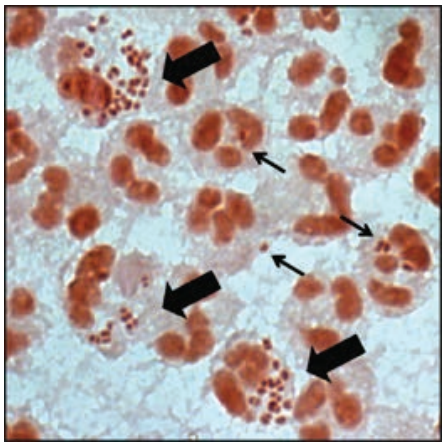


Figure 1. Gram stain of a Gc-infected patient urethral exudate showing Gc associated with neutrophils. Gc can be seen inside and associated with neutrophils. Not all neutrophils have Gc associated. From Johnson and Criss *Front. Microbiol.*, 2:77, 2011. doi:10.3389/fmicb.2011.00077.

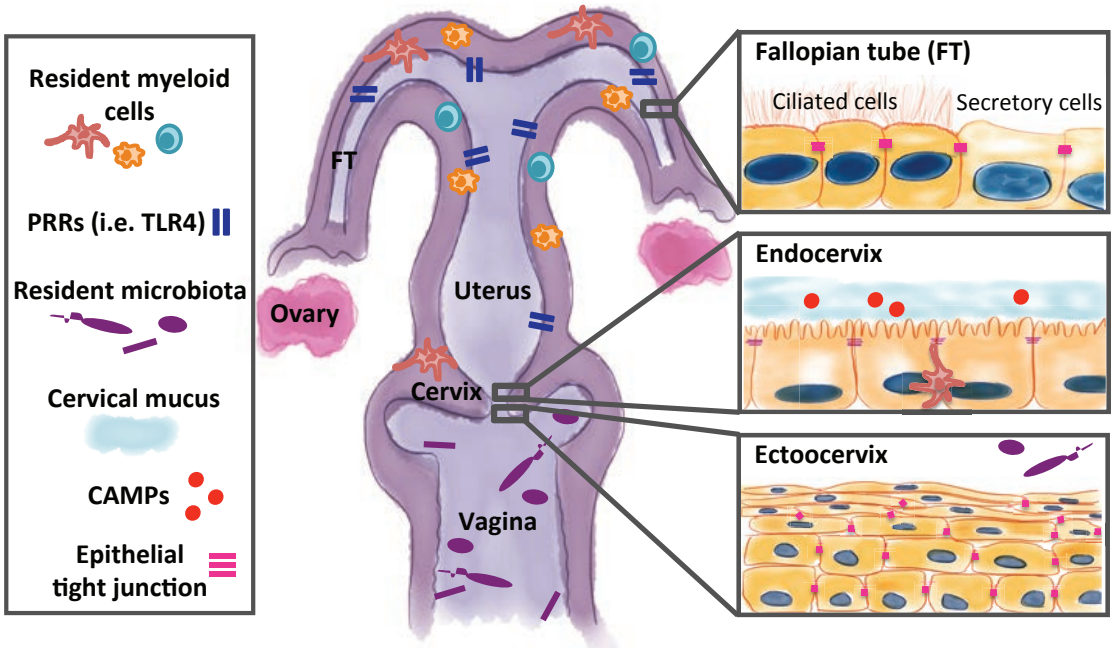


Figure 2. Gc initially infects the endocervix, a transition from multilayered squamous epithelium lining the lower FRT to single columnar epithelium lining the upper FRT. Epithelial cells provide multiple barriers to infection including maintaining epithelial tight junctions and barrier integrity, producing thick cervical mucus, and secreting cationic antimicrobial peptides (Quayle, 2002). Gonococcal factors contributing to survival from cationic antimicrobial peptides in the genital tract include expression of the MtrCDE efflux pump, modification of lipid A with phosphoethanolamine (refer to Table 1 for more information) (Hobbs et al., 2013; Jerse et al., 2003; Kandler et al., 2014), and the MisR-MisS two-component regulatorysystem (Gangaiah et al., 2017; Kandler et al., 2016). The transition from the lower to the upper female reproductive tract is also marked by changes in microbiota, pattern-recognition receptor expression, and myeloid cell frequency (Wira et al., 2005). Figure and figure legend from Stevens and Criss *Curr Opin Hematol*. 2018. doi: 10.1097/MOH.0000000000000394.

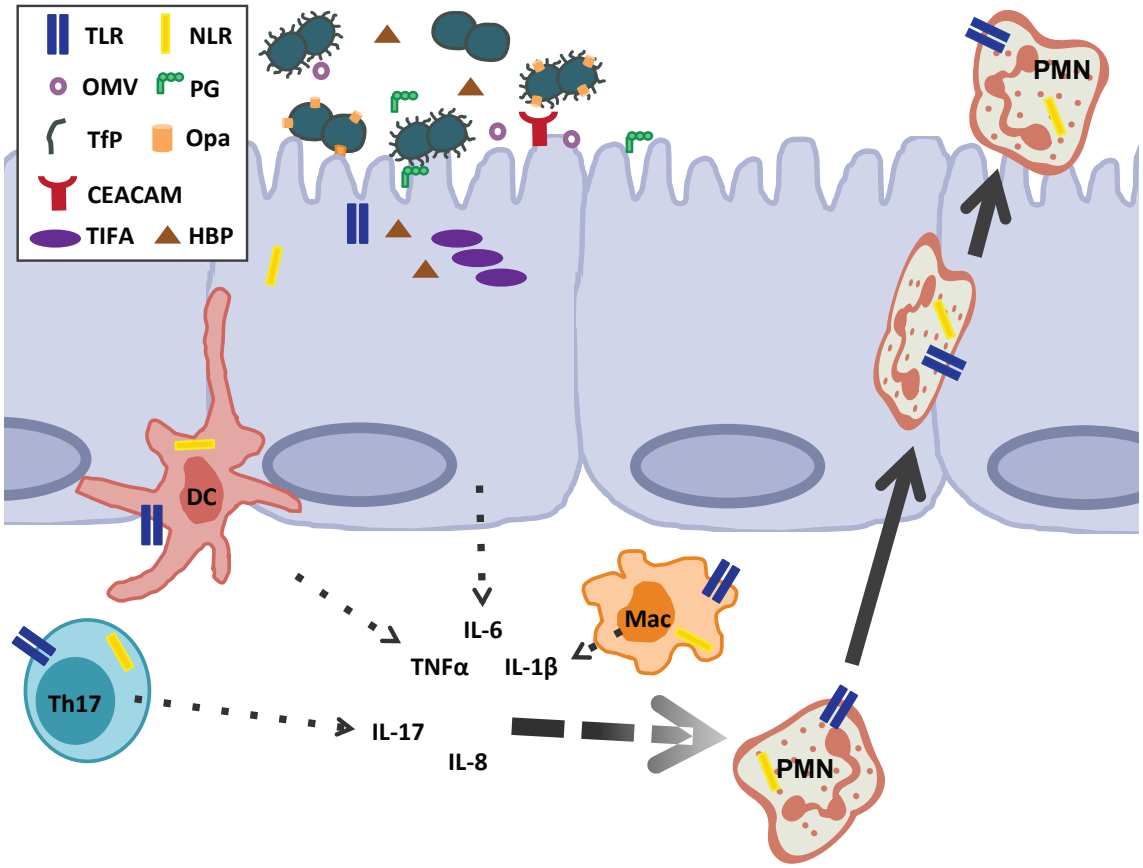


Figure 3. Epithelial and resident immune cells initiate the immune response to Gc. Important gonococcal surface structures include opacity-associated proteins (Opa), the type IV pilus (TfP), lipooligosaccharide, and porin. During infection, there is a mixture of gonococci with varied expression of Opa proteins, lipooligosaccharide type, and the type IV pilus. Opa proteins interact with CEACAMs on host epithelial and immune cells such as neutrophils (PMN). Pattern-recognition receptors on epithelial and resident immune cells such as PMNs, macrophages (Mac), dendritic cells (DC), and Th17 cells recognize pathogen-associated molecular patterns presented on the surface of Gc and/or released from the bacterium freely or as components of outer membrane vesicles (OMV). In addition to well known pathogen-associated molecular patterns such as lipooligosaccharide and peptidoglycan (PG), the gonococcal cell envelope and OMVs

may harbor additional factors that affect the host response to infection (Zielke et al., 2014). Cervical epithelial cells respond to Gc through stimulation of Toll-like receptor (TLR2) but not TLR4, whereas resident immune and upper FRT cells, including Fallopian tube epithelial cells, are particularly poised to respond to lipooligosaccharide via TLR4 (Fazeli et al., 2005; Fichorova et al., 2002). Gonococcal porin and other lipoproteins stimulate TLR2 to activate NF-kB-driven inflammatory cytokine production (Fisette et al., 2003; Massari et al., 2002). TLR4 is potently activated by the lipid A portion of lipooligosaccharide from Gc and its close relative, *Neisseria meningitidis* (John et al., 2017). PG is primarily recognized by intracellular NOD-like receptors (NLRs) (Chan and Dillard, 2017; Mavrogiorgos et al., 2014). An additional mechanism of intracellular detection of Gram-negative bacteria is the TRAF-interacting protein with forkhead-associated (TIFA) domain-dependent detection of heptose-1,7-bisphosphate (HBP), an intermediate in lipooligosaccharide production (Gaudet and Gray-Owen, 2016; Gaudet et al., 2017; Gaudet et al., 2015). In response to Gc, epithelial and resident myeloid cells secrete proinflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-8 and sentinel Th17 cells release IL-17. These cytokines establish a gradient that serves to recruit neutrophils from the bloodstream and activate them at the site of infection. Figure legend from Stevens and Criss *Curr Opin Hematol*. 2018. doi: 10.1097/MOH.0000000000000394.

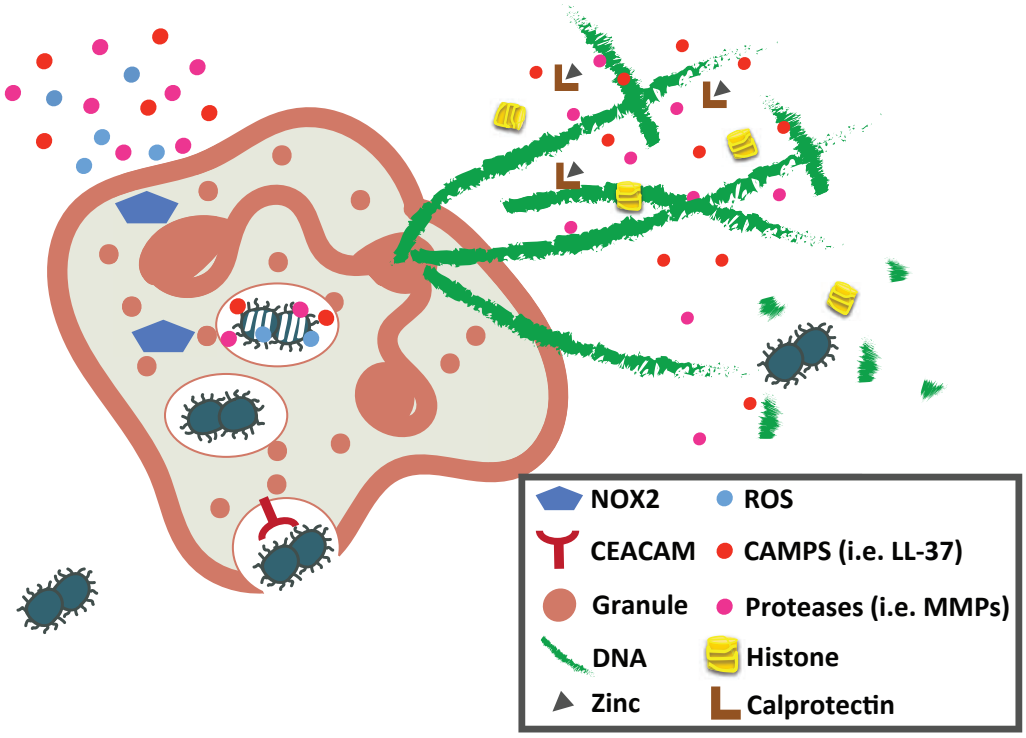


Figure 4. Neutrophils possess an arsenal of extracellular and intracellular killing mechanisms that Gc can evade, potentially leading to off-target host cell damage. Neutrophil killing mechanisms include phagocytosis, production of reactive oxygen species, degranulation, and neutrophil extracellular trap (NET) formation. Granule components include cationic antimicrobial proteins (CAMPs) (i.e., LL-37), membrane subunits of the nicotinamide adenine dinucleotide phosphate oxidase 2 (NOX2), and proteases (i.e., matrix metalloproteinases, MMPs). NETs are comprised of DNA that is associated with histones, CAMPs, some proteases, and calprotectin. *N. gonorrhoeae* has evolved elegant mechanisms to resist neutrophil killing (Criss and Seifert, 2012; Johnson and Criss, 2011). Neutrophil extracellular trap and associated granule component evasion strategies include resistance to antimicrobial compounds, a gonococcal thermonuclease, acquisition of zinc from calprotectin by TdfH, and maintenance of envelope integrity (Gunderson and Seifert, 2015; Handing and Criss,

2015; Jean et al., 2016; Juneau et al., 2015; Ragland et al., 2017; Stohl et al., 2013). Variation in Opa protein expression can affect neutrophil activation and gonococcal survival (Ball and Criss, 2013; Johnson et al., 2015; Johnson and Criss, 2013; Sarantis and Gray-Owen, 2007, 2012; Sintsova et al., 2014; Smirnov et al., 2014). As Gc can evade neutrophil killing, reactive products generated by neutrophils are instead poised to induce host cell damage. Figure legend from Stevens and Criss *Curr Opin Hematol*. 2018. doi: 10.1097/MOH.0000000000000394.

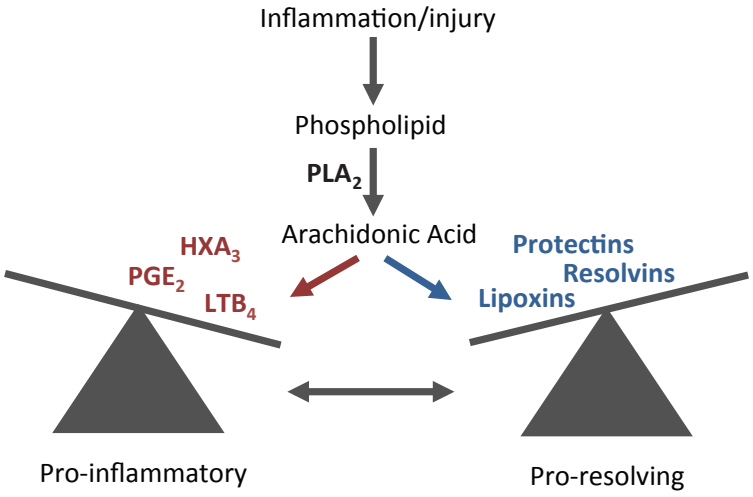


Figure 5. Pro-inflammatory and pro-resolving eicosanoids contribute to homeostasis and this balance can be disrupted during infection/inflammation. In response to inflammation, infection, or injury, PLA_2 liberates arachidonic acid, which can be converted to eicosanoids. Pro-inflammatory eicosanoids include HXA_3, LTB_4, and PGE_2. These are balanced during homeostasis and resolution of inflammation by resolvins, protectins, and lipoxins.

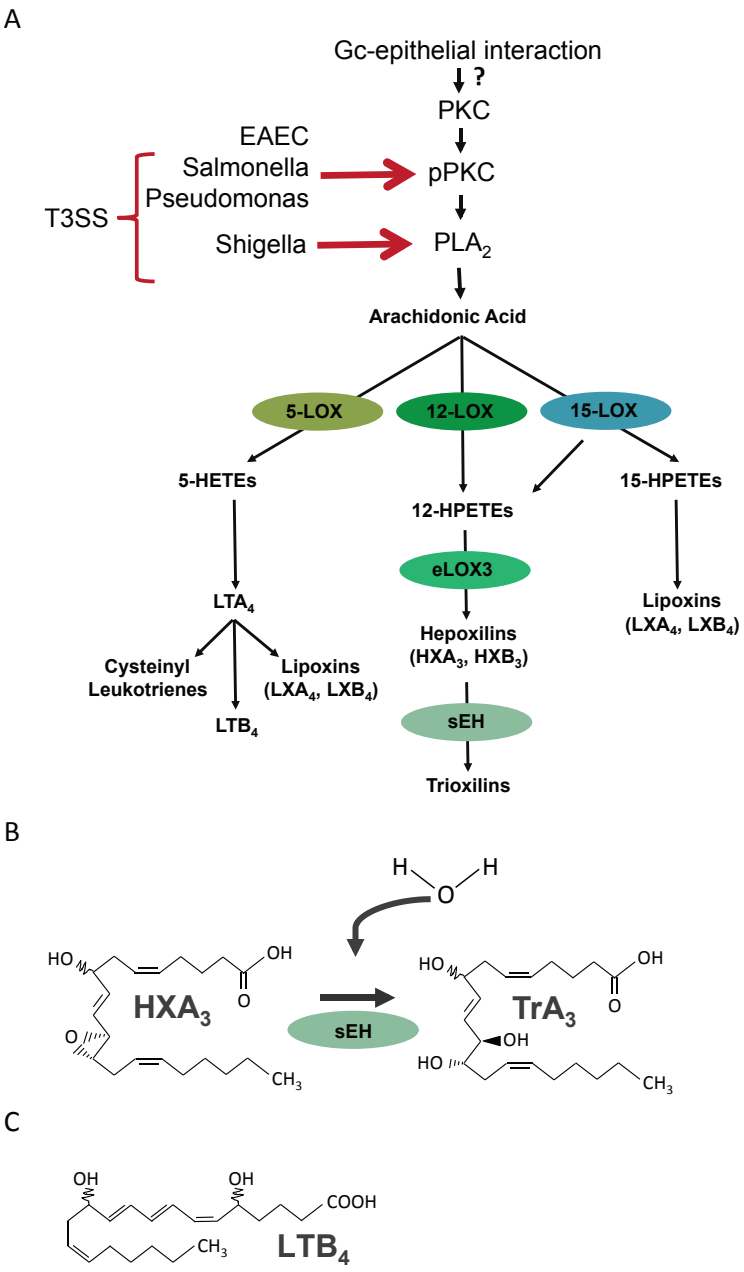


Figure 6. Schematic of lipoxygenase-mediated generation of eicosanoids from AA. Adapted from Harizi et al. (Harizi et al., 2008). 12-lipoxygenase activity can be exhibited by 12- and 15-lipoxygenase isoforms. HXA_3 is produced via 12-lipoxygenase activity, while LTB_4 is produced via 5-lipoxygenase activity, in conjunction with FLAP and LTA_4 hydrolase (not pictured here). Liberation of AA by PLA_2 is the rate-limiting step. HXA_3 is converted to an inert (non-chemotactic to neutrophils) TrA_3 by sEH.

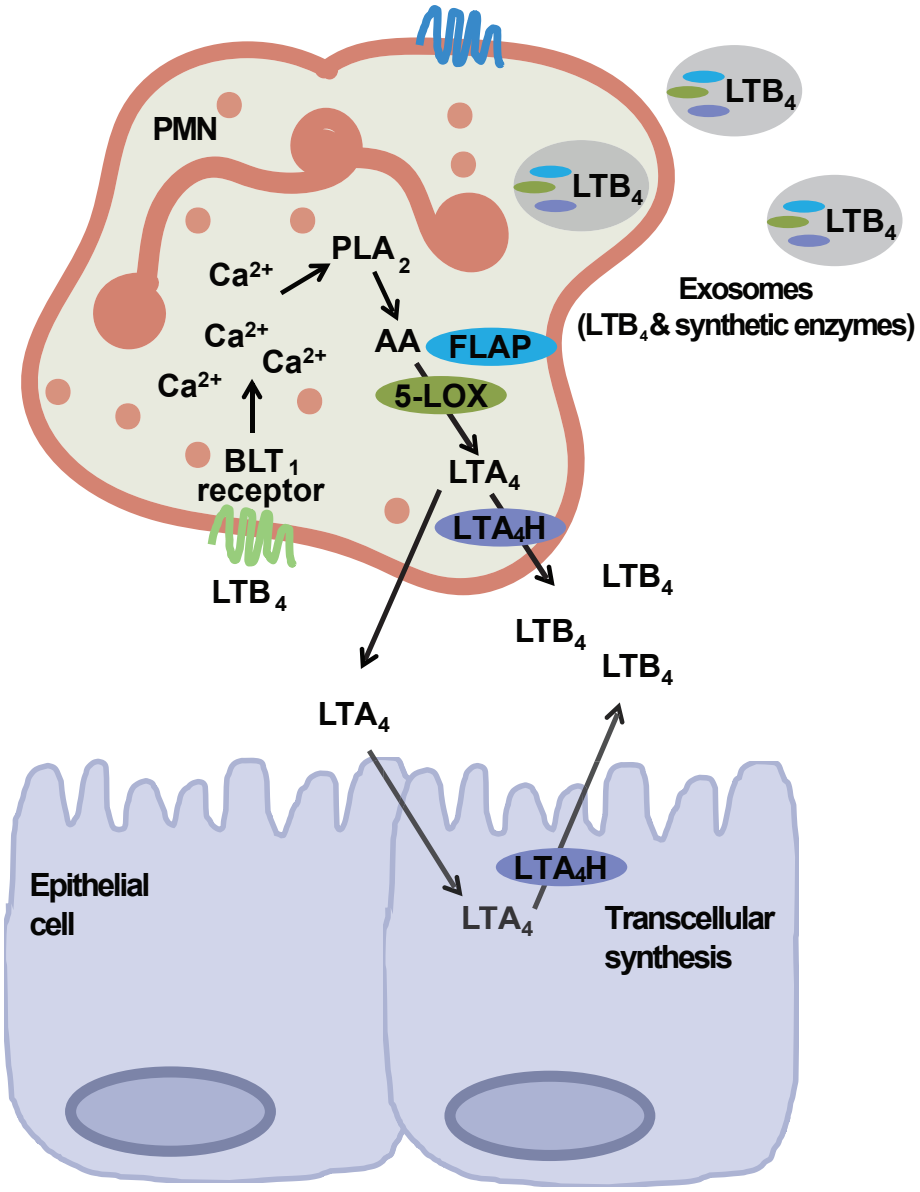


Figure 7. Generation of LTB_4 in neutrophils. Upon stimulus, including LTB_4 binding its high affinity BLT_1 receptor on neutrophils, the resultant calcium-flux stimulates PLA_2-dependent liberation of AA. 5-lipoxygenase translocates to the nuclear membrane where, in conjunction with FLAP and LTA_4 hydrolase, it converts AA to LTB_4.

Protein Name	Mechanisms of Resistance to Neutrophils	References
Mpg/NGO1686	PG peptidase enhances resistance to CAMPs and ROS via presentation of type IV pili	(Stohl et al., 2013), (Stohl et al., 2005), (Stohl et al., 2012)
RecN	Unclear	(Stohl et al., 2005)
LtgA, LtgD	PG lytic transglycosylases together confer envelope integrity to defend Gc from lysozyme and neutrophil elastase; limit granule exocytosis and phagosome-granule fusion	(Ragland et al., 2017)
TdfH	TonB-dependent transporter extracts Zn from calprotectin and enhances Gc survival in NETs	(Jean et al., 2016)
Nuc	Thermonuclease released extracellularly cleaves NETs	(Juneau et al., 2015)
LptA	Phosphoethanolamine transferase for lipid A enhances Gc resistance to neutrophil CAMPs and proteases; limits phagosome-granule fusion	(Handing and Criss, 2015)
MIP	Peptidyl-prolyl isomerase protects by unknown mechanism	(Reimer et al., 2016)
LdhA, LdhD	D-lactate dehydrogenases enhance Gc survival in neutrophils, potentially by obtaining lactate as a carbon source	(Atack et al., 2014)
Pili	Type IV pili protect against CAMPs and ROS	(Stohl et al., 2013)
Lst	Sialylation of Gc N-lactotetraose-containing LOS inhibits phagocytosis by neutrophils in suspension	(Kim et al., 1992; Rest and Frangipane, 1992)
PorB	Essential porin inhibits the neutrophil oxidative burst	(Chen and Seifert, 2011; Lorenzen et al., 2000)

Table 1. Proteins of *N. gonorrhoeae* that confer resistance to neutrophils. From Stevens and Criss *Curr Opin Hematol*. 2018. doi: 10.1097/MOH.0000000000000394.

2. Chapter 2: Endocervical and neutrophil lipoxygenases coordinate neutrophil transepithelial migration to *Neisseria gonorrhoeae*

This chapter is a modified version of the previously published article, "Endocervical and neutrophil lipoxygenases coordinate neutrophil transepithelial migration to *Neisseria gonorrhoeae*" [J Infect Dis. 2018 Jun 13. doi: 10.1093/infdis/jiy347].

2.1 Introduction

Gonorrhea is caused by the human-specific pathogen *Neisseria gonorrhoeae* (Gc) and is an important sexually transmitted infection worldwide, with 78 million cases estimated per year (Wi et al., 2017). Lack of a protective immune response, emergence of drug-resistant strains, and lack of a vaccine have prompted the classification of Gc as an urgent threat (Wi et al., 2017). The hallmark of Gc infection is robust influx of polymorphonuclear leukocytes (neutrophils or PMNs) (Johnson and Criss, 2011). Gc evades a protective adaptive immune response and instead skews the immune response towards neutrophilic inflammation (Stevens and Criss, 2018). While male urethritis commonly presents with symptoms, up to 80% of female cervical infections are asymptomatic (Edwards and Apicella, 2004). As a result, clinical complications are most common and serious in women. Cervicitis still occurs in women with asymptomatic infection and despite neutrophil recruitment, Gc can evade neutrophil killing and is not cleared (Criss and Seifert, 2012; Johnson and Criss, 2011). Sustained neutrophil influx that does not clear infection contributes to severe clinical sequelae including pelvic inflammatory disease (PID), tubal scarring, ectopic pregnancy, and infertility. Although, gonococcal infection and resulting neutrophilic inflammation cause significant morbidity

in women, to date the mechanisms underlying Gc-cervical cell interactions that stimulate neutrophil influx are poorly understood.

During Gc infection, mucosal epithelial and resident immune cells secrete cytokines including IL-8 that recruit neutrophils from the bloodstream into infected tissue (Criss and Seifert, 2012). These cytokines may not be sufficient for the terminal step of neutrophil migration across the epithelium into the apical/luminal compartment, termed neutrophil transmigration (McCormick et al., 1995). Instead, mucosal epithelial cells release additional chemotactic factors that drive transmigration (McCormick et al., 1998; Mrsny et al., 2004). Recent work has identified an important role for eicosanoids, including during neutrophil migration to mucosal bacterial pathogens (Bhowmick et al., 2013; Boll and McCormick, 2012; Boll et al., 2012a; Boll et al., 2012b; Hurley et al., 2011; Hurley et al., 2004; McCormick et al., 1995; Mrsny et al., 2004; Mumy et al., 2008; Pazos et al., 2008; Pazos et al., 2017; Pazos et al., 2015; Szabady and McCormick, 2013; Tamang et al., 2012; Yonker et al., 2017; Zurawski et al., 2006). Eicosanoids are arachidonic acid (AA)-derived bioactive lipids (Dennis and Norris, 2015). The rate-limiting step in eicosanoid generation is liberation of AA by phospholipase A_2 (PLA_2) (Dennis and Norris, 2015). AA can then be oxidized by lipoxygenases (LOX) or cyclooxygenases (COX) to generate eicosanoids, including hepoxilins, leukotrienes, and prostaglandins (Dennis and Norris, 2015). Of these, hepoxilin A_3 (HXA_3) and leukotriene B_4 (LTB_4) are particularly potent neutrophil chemoattractants that coordinate neutrophil migration to sites of bacterial infection and inflammation (Le Bel et al., 2014; Nemeth and Mocsai, 2016; Szabady and McCormick, 2013). HXA_3 is produced by different 12-lipoxygenase enzymes that are cell-type specific (Mashima and Okuyama, 2015). Neutrophil chemotactic activity of HXA_3 requires its epoxide ring, which is rapidly degraded by an acidic environment and/or endogenous soluble epoxide hydrolase (sEH) (Morisseau et al., 2012; Pace-Asciak, 2015). LTB_4 is produced from AA by sequential

activity of 5-lipoxygenase (5-LOX) and LTA_4-hydrolase (Rouzer et al., 1986). LTB_4 is primarily produced by myeloid cells and amplifies neutrophil influx in a number of inflammatory disease processes (Le Bel et al., 2014; Nemeth and Mocsai, 2016). Recent work has revealed an interplay between bacterial promotion of eicosanoid production and eicosanoid regulation of innate immune responses in the context of host-pathogen interactions (Le Bel et al., 2014; Pazos et al., 2017).

Neutrophilic inflammation is associated with a number of mucosal infections with pathogenic bacteria including *Salmonella, Shigella flexneri,* Enteroaggregative *Escherichia coli, Streptococcus pneumoniae,* and *Pseudomonas aeruginosa*, which use HXA_3 and/or LTB_4 to coordinate neutrophil transepithelial migration (Bhowmick et al., 2013; Boll and McCormick, 2012; Boll et al., 2012a; Boll et al., 2012b; Hurley et al., 2004; Mrsny et al., 2004; Pazos et al., 2017; Pazos et al., 2015; Tamang et al., 2012; Zurawski et al., 2006). Each of these pathogens stimulates eicosanoid production in a distinct way, suggesting that individual bacteria have evolved unique mechanisms to co-opt eicosanoid-driven neutrophilic inflammation. In contrast, the signals driving neutrophil influx to gonococcal infection are poorly understood. This is particularly problematic in the context of female infection, where unresolved infection and sustained neutrophilic inflammation contribute to severe clinical consequences. In this work we established a three-component Gc-endocervical cell-neutrophil model, and found evidence to support the hypothesis that eicosanoids are required for the coordination of neutrophil transepithelial migration in response to infection.

2.2 Materials and Methods

2.2.1 *Bacterial strains and growth conditions*

A piliated, OpaD-expressing derivative of Gc strain FA1090 (Ball and Criss, 2013) was used for these studies unless otherwise indicated. Gc was maintained on Gonococcal Medium Base (GCB) (BD Difco) with Kellogg's supplement I + II (Kellogg et al., 1963). For infection of epithelial cells, 16-18 hour overnight lawns were cultivated at 37^{o}C in 5% CO_2, swabbed into liquid medium, and resuspended in Hank's balanced salt solution (HBSS; with Ca^{2+} and Mg^{2+}; Thermo Scientific) with 10 mM HEPES pH 7.4 and 5 mM $NaHCO_3$ ($HBSS^{+}$) at a concentration of 7.6 x 10^7 CFU/mL (target MOI=10). For trypsin-treatment of Gc, 5 x 10^8 CFU were treated with 40 µg/mL Sequencing grade Trypsin (Promega) or vehicle (Trypsin resuspension buffer, Promega) for 1hr at 37^{o}C on a rotating drum. Trypsin was quenched with Roswell Park Memorial Institute medium (RPMI) containing 10% FBS, washed in PBS, and resuspended in $HBSS^{+}$ for infection.

2.2.2 *Human neutrophil isolation*

Venous blood was collected from healthy human donors who provided informed consent. All human subject research was conducted in accordance with a protocol approved by the University of Virginia Institutional Review Board for Health Sciences Research. Red blood cells were removed from heparinized blood by dextran sedimentation and neutrophils were isolated over a Ficoll-Hypaque gradient as previously described (Stohl et al., 2005) and resuspended in Dulbecco's phosphate buffered saline (DPBS; without Ca^{2+} and Mg^{2+}; Thermo Scientific) with 0.1% dextrose. Neutrophils were kept on ice and used within 1h following preparation. Neutrophil

preparations were assessed by phase-contrast microscopy and contained >95% neutrophils. Replicate experiments used neutrophils from different donors on separate days.

2.2.3 *Cell culture*

Human End1/E6E7 (End1) cells (ATCC® CRL-2615™) were maintained and polarized End1 monolayers established on inverted Corning™ Transwell ™ inserts as described previously (Fichorova et al., 2005), with the following details. Human End1/E6E7 (End1) cells were acquired from the ATCC® (CRL-2615™) and are a well-differentiated endocervical epithelial cell line generated from an endocervical tissue specimen obtained from a premenopausal woman undergoing hysterectomy for endometriosis (Fichorova and Anderson, 1999). End1 cells were maintained in keratinocyte serum-free medium (KSFM, Life Technologies) supplemented with provided bovine pituitary abstract (BPE, 50 µg/mL) and recombinant epidermal growth factor (EGF, 0.1 ng/mL) as well as 0.4 mM $CaCl_2$ and 1X Antibiotic-Antimicotic (ThermoFisher Scientific). End1 cells were maintained at 37°C, 5% CO_2 in a humidified chamber and routinely tested negative for mycoplasma.

To establish polarized monolayers, End1 cells were seeded on inverted 6.5 mm diameter Corning™ Transwell ™ inserts with 3-µm pores (ThermoFisher Scientific) coated with 5 µg/cm^2 human type IV collagen (Sigma). 7 x 10^4 End1 cells were seeded on each coated inverted insert and allowed to attach for 5 hours before reverting into a 24-well tissue culture plate containing KSFM and incubated at 37°C, 5% CO_2 for 8-11 days, after which time transepithelial electrical resistance (TEER) measured using an EVOM^2 voltometer (World Precision Instruments, Inc.) was ≥ 150 Ω cm^2 (Fichorova et al., 2005; Sathe and Reddy, 2014). Barrier function of End1 monolayers was also

monitored by stable fluid resistance between apical and basolateral reservoirs and by measurement of 10 kDa FITC-dextran (Sigma) flux in the apical and basolateral compartments of End1 monolayers using a black F96 MicroWell™ (ThermoFisher Scientific) and Wallac Victor-2 1420 Multilabel Counter 485/535nm (Perkin-Elmer).

2.2.4 *Microscopy*

End1 monolayers on Transwell™ inserts were fixed in 4% paraformaldehyde (Electron Microscopy Sciences), permeabilized in 0.01% Triton-X 100 (ThermoFisher Scientific) and blocked in 10% normal goat serum in phosphate-buffered saline for 1 hr at room temperature. ZO-1 was detected using a purified mouse anti-human ZO-1 monoclonal antibody (BD Biosciences) followed by an Alexa-Fluor 488-coupled goat anti-mouse immunoglobulin G (ThermoFisher Scientific). Gc was labeled using a polyclonal rabbit anti-Gc antibody (Biosource) followed by an Alexa-Fluor 488-coupled goat anti-mouse immunoglobulin G (ThermoFisher Scientific). Phalloidin-555 and DAPI were used to stain actin and nuclei respectively. Confocal images were captured on a Zeiss LSM-700 confocal laser scanning microscope at the Advanced Microscopy Facility (AMF) at the University of Virginia. Z-stacks were captured and exported from Zen Black Edition (Zeiss) as TIF files. For scanning electron micrographs (SEM), End1 monolayers on inverted Transwell™ inserts were fixed overnight at 4^{o}C in 4% paraformaldehyde/2% glutaraldehyde (Electron Microscopy Sciences) and then transferred to the AMF at UVA for processing before images were acquired on a Zeiss SEM at the UVA AMF.

2.2.5 *PMN Transmigration assays*

End1 monolayers were washed and inverted into a humidified chamber. Monolayers were apically infected with 2.3 x 10^6 Gc CFU equivalents (MOI=10) for 1 hr (unless otherwise indicated) at 37°C, 5% CO_2. To assay neutrophil migration across an uninfected End1 monolayer with Gc in the apical reservoir, Gc were added to the bottom of a tissue culture well for 1 hr, followed by addition of an End1 monolayer on a Transwell™ insert immediately prior to addition of neutrophils. Neutrophil migration was assayed as previously described (Parkos et al., 1991), with the following details. Following infection, monolayers were washed extensively in $HBSS^+$ to remove non-adherent bacteria and reverted into 24-well tissue culture wells containing 1 mL $HBSS^+$ per well. 1 x 10^6 primary human neutrophils were added to the basal reservoir of Transwell™ inserts and incubated for 2 hours at 37°C, 5% CO_2. The number of neutrophils transmigrated was measured using a colorimetric assay for the neutrophil primary granule component myeloperoxidase (MPO) using ABTS™ Chromophore Diammonium Salt (EMD Millipore), read on a Wallac Victor-2 1420 Multilabel Counter (Perkin-Elmer) using 1s readings at 405nm, and quantified relative to a standard curve for each donor and each experiment. For all PMN transmigration assays, migration was compared to buffer alone as a negative control over two hours or an imposed apical gradient of fMLP (1 μM) over two hours as a positive control for neutrophil migratory capacity. In all experiments, > 1 x 10^5 neutrophils underwent transmigration to fMLP. For each experiment, adhered CFU were enumerated by incubating infected monolayers with 1% Saponin, 5 mM $MgSO_4$ for 15 minutes at 37°C, 5% CO_2, disrupting membranes, and plating serial dilutions on GCB agar (Table 2).

2.2.6 *ELISA*

Supernatants from Gc-infected End1 monolayers +/- neutrophils were collected, passed through a 0.2 µm filter, and stored at -80°C until processed. ELISAs for IL-8 (R&D Systems) and LTB_4 (Cayman Chemical) were performed per manufacturers' protocols.

2.2.7 *Inhibitor and drug treatments*

End1 monolayers were pre-treated with inhibitors or vehicle controls diluted in $HBSS^+$, incubated at 37°C, 5% CO_2 for the indicated times. Monolayers were then thoroughly washed in $HBSS^+$ before infection. The inhibitors used did not affect Gc viability or adherence to End1 monolayers, as assayed by CFU enumeration after saponin lysis, serial dilution, and plating on GCB agar (Table 2). , End1 and neutrophil viability, as monitored by Trypan Blue (HyClone) exclusion, was >95% after treatment with inhibitor or vehicle. In all cases, inhibitor or vehicle treatment did not affect neutrophils' ability to migrate towards an imposed apical gradient of fMLP.

Epithelial cell treatments: Inhibitors used to pre-treat End1 monolayers were as follows: 5 µM Chletherine chloride (CCL; Enzo Life Sciences), 5 µM ONO-RS-081 (Enzo Life Sciences), 6µM $cPLA_2$α-inhibitor (Millipore Sigma), 7 µM $sPLA_2$-inhibitor 2,4'-Dibromoacetophenone (Sigma), 50µM Caffeic acid (Sigma), 25 µM Zileuton (Cayman Chemical), 50 µM Cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC) (Enzo Life Sciences), 100 µM MK571 (Fisher Scientific), and 50 µM NS398 (Cayman Chemical). Epithelial cells were treated with inhibitors diluted in $HBSS^+$ for 1-2 hr at 37°C, 5% CO_2. Following inhibitor treatment, End1 monolayers were extensively washed in $HBSS^+$ prior to infection and neutrophil migration.

Drug treatments during PMN transmigration: For IL-8 blocking experiments, 20 µg/mL IL-8 blocking antibody (R&D Biosystems) was added to both the apical and basolateral reservoirs of infected End1 inserts or to control wells. For assays of BLT_1 antagonism, 2.5, 0.625, or 0.156 µg/mL LY223982 (Cayman Chemical) was added to the apical reservoir of infected End1 inserts or to control wells. For the soluble epoxide hydrolase (sEH) sensitivity assay, 100 or 200 µg/mL recombinant human sEH was added to the apical reservoir of infected End1 inserts or control wells 10 minutes prior to and throughout neutrophil transepithelial migration. The human sEH was produced in an insect cells/baculovirus expression system as previously described (Beetham et al., 1993). The targeted enzyme was purified by affinity chromatography (Wixtrom et al., 1988). The human sEH preparation was 97%, pure, as judged by SDS-PAGE analysis and scanning densitometry. The activity of the purified enzyme was tested using a radioactive surrogate substrate (Morisseau and Hammock, 2007). For the assay of neutrophil transepithelial migration sensitivity to 5-LOX inhibition, neutrophils were pre-incubated with 25 µM Zileuton or equivalent vehicle at 37^{o}C for 1hr in $HBSS^{-}$, washed, and re-suspended in $HBSS^{+}$ with 25 µM Zileuton or equivalent vehicle for neutrophil transepithelial migration.

2.2.8 *shRNA knockdown*

A lentiviral delivery system was used to mediate shRNA knockdown of 12R-LOX, eLOX-3, or 5-LOX in End1 cells. MISSION^{TM} (Sigma Aldrich) shRNA clones for GenBank^{TM} accession number, NM_001139.2-2340s21c1 (12R-LOX), NM_021628.1-1945s1c1 (eLOX-3), and NM_000698.1-405s1c1 (5-LOX) were packaged in a pKLO.1-Puro lentivirus vector, with both bacterial (ampicillin) and mammalian (puromycin) resistance markers. Plasmids were prepared from bacterial glycerol stocks (Sigma),

selected in LB broth containing 100 µg/mL ampicillin, and purified using a QIAprep Spin Miniprep Kit (Qiagen) per the manufacturer's protocol. Plasmids were packaged in 293T cells and collected lentivirus used to infect End1 cells. Stable knockdown was selected for using 1 µg/mL puromycin.

2.2.9 *qRT-PCR*

RNA was extracted from three biological replicate End1 monolayers or HL60 cells per condition using a Qiagen RNeasy Plus Mini Kit (Qiagen) per manufacturer protocol. The primers used in the quantitative real-time PCR (qRT-PCR) are as follows: *gapdh* forward: 5'-CCCATCACCATCTTCCAGGA-3', reverse: 5'-GTTGTCATGGATGACCTTGG-3' (Tamang et al., 2012); *alox12* forward: 5'-AAGCCCAAAGCTGTGCTAAA-3', reverse: 5'-TGCAGCAGGAGAGCTGAGTA-3' (Tamang et al., 2012); *alox15* forward: 5'-GAACTCAAGGTGGAAGTACC-3', reverse: 5'-CTTCAGGCAGGCTCAGGACG-3' (Tamang et al., 2012); *alox12b* forward: 5'-ACCGTGCAGTGCCCTCAGGA-3', reverse: 5'- CCGGAGTGCCAGGGTCTCGT-3' (Tamang et al., 2012); *aloxe3* forward: 5′-GAGCAAAAATCTCGCCAGTC-3′, reverse: 5′-GGGCTTTGTCTCAGAAATCG-3′ (Kretz et al., 2013). For *alox5* and *ltb4r1*, pre-designed KiCqStart®SYBR® Green Primers (Sigma, KSPQ12012) were as follows: *alox 5* forward: 5'-AAATGCCACAAGGATTTACC-3', reverse: 5'-ATCGCTTTGGAGTAATTCAG-3'; *ltb4r* forward: 5'-AATATCCACATGCAACATCC-3', reverse: 5'-GGATCTCCTCTTCTTAGGTC-3'. A one-step qRT-PCR reaction was run on an ABI 7500 sequence detection system (Applied Biosystems) and data collected using ABI Sequence Detection software, version 1.2 (Applied Biosystems). Data were normalized to *gapdh* expression, analyzed using the comparative cycle threshold (C_T)

method, and then target gene expression level compared using relative quantification (Biosystems, 1997).

2.2.10 *Lipid extractions*

Lipids were isolated from End1 supernatants using solid phase extraction as previously described (Pazos et al., 2015; Tamang et al., 2012). Non-polarized End1 cells in 162-cm^2 flasks were infected with Gc at an MOI=100 in $HBSS^+$ or mock infected for 1 hr. Cells were then washed three times with $HBSS^+$ and incubated a further two hours in 15 mL $HBSS^+$. Infection supernatants were then collected, acidified to a pH 5, and extracted by solid-phase extraction using a Sulpeco Discovery DSC-18 SPE column (Sigma) and eluted with 3 mL methanol (Pazos et al., 2015; Tamang et al., 2012). Samples were then dried under nitrogen gas and stored at -80^oC until used. Prior to use, samples were re-suspended in $HBSS^+$.

2.2.11 *Statistical analysis*

For neutrophil transmigration assays, results are expressed as a mean ± standard error of the mean for ≥ three independent experiments, with three transwells per condition. Statistics were calculated using a two-tailed, Student *t* test, either unpaired for neutrophil migration and qRT-PCR or paired for ELISA and transmigration to lipid-extracted infection supernatants (Excel). For analysis of MOI-dependent neutrophil transmigration, statistics were calculated using a One-way ANOVA with Tukey *post-hoc* test (GraphPad Prism). In all cases a P value < 0.05 was considered statistically significant compared either to the negative control (buffer alone) or between groups as indicated.

2.3 Results

2.3.1 *Contact-dependent Gc infection of polarized endocervical cell monolayers stimulates neutrophil migration*

To define factors governing neutrophil migration in response to Gc infection, we established an *in vitro* three-component system to model infection and neutrophil migration at the endocervix (Figure 8A). End1 cells maintain stable expression of endocervical differentiation markers and upregulate inflammatory biomarkers when exposed to Gc (Fichorova et al., 2002; Fichorova et al., 2001), providing a physiologically relevant cell line to model human endocervix. End1 monolayers exhibited apicobasal polarity and junctional integrity, as reflected by increased transepithelial electrical resistance (Figure 9A), inhibition of paracellular flux of 10 kDa FITC-dextran in a divalent cation-dependent manner (Figure 9B), and lateral localization of the tight junctional protein ZO-1 (Figure 9C). These findings agree with previous reports using polarized End1 cells (Fichorova et al., 2005; Sathe and Reddy, 2014). A piliated, OpaD-expressing derivative of FA1090 Gc avidly colonized the apical surface of inverted End1 monolayers 1 hour after inoculation (Figure 9D). Primary human neutrophils were then added to the basal aspect of End1 monolayers and transepithelial migration into the apical reservoir was measured as cell equivalents of neutrophil myeloperoxidase. Gc infection elicited neutrophil transepithelial migration over 2 hours that increased with increasing bacterial inoculum (Figure 8B). In all experiments, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) was included as a positive control, and buffer alone (no apical stimulus) as a negative control (Figure 8B). MOI-dependent neutrophil migration to Gc was also observed after 4 hours of apical infection at lower inocula (Figure 10A),

and migration was similar between infection with an MOI of 10 after 1 hour and an MOI of 3 after 4 hours (Figure 10B). After 1 hour of infection at MOI of 10, migration that was significantly greater than the negative control was first observed 75 minutes after adding neutrophils and increased at times thereafter (Figure 8C). The following experiments were performed with 1 hour Gc infection at an MOI of 10 and 2 hours neutrophil migration, unless otherwise noted. Neutrophils that had migrated across Gc-infected End1 monolayers appeared highly polarized and protrusive by scanning electron microscopy (Figure 9E).

We hypothesized that Gc-endocervical cell contact is required to stimulate neutrophil migration and found two lines of evidence in support of this hypothesis. First, there was no neutrophil transepithelial migration when Gc was in the apical reservoir but not in contact with the End1 monolayers (Figure 8D). Under these conditions, Gc did not associate with the End1 apical surface as assessed by CFU enumeration (Table 2) and confocal microscopy (Figure 11). Second, neutrophils did not migrate across acellular inserts towards an equivalent CFU of Gc (Figure 8D). In both conditions, neutrophils still migrated towards fMLP; migration across the End1 monolayer was greater than that observed across an acellular insert, likely because the presence of the monolayer helped maintain a concentration gradient of fMLP. These results show that polarized endocervical cells are necessary and sufficient to coordinate the migration of neutrophils after apical infection with Gc.

2.3.2 *Neutrophil transepithelial migration to Gc requires neutrophil-derived leukotriene B_4*

Given that human gonococcal infection is characterized by neutrophil recruitment, we examined the possibility that the eicosanoid neutrophil chemoattractant LTB_4 is involved. LTB_4 accumulated over time in the apical medium of Gc-infected End1

monolayers following neutrophil transepithelial migration (Figure 12A). LTB_4 concentrations were significantly higher in apical compared to basal medium after 2 hours, suggesting establishment of an apical-to-basolateral gradient of LTB_4 following infection (Figure 12A). To evaluate if LTB_4 contributed to neutrophil transepithelial migration, LY223982, an antagonist to the high-affinity LTB_4 receptor BLT_1, was added to the apical medium (Pazos et al., 2015). LY223982 significantly inhibited neutrophil transepithelial migration following Gc infection in a concentration-dependent manner (Figure 12B). LTB_4-BLT_1 interaction increases the liberation of AA to consequently stimulate more LTB_4 production (Le Bel et al., 2014; Nemeth and Mocsai, 2016). These results show that LTB_4 is released during neutrophil migration across Gc-infected End1 monolayers, and implicate LTB_4 signaling through BLT_1 as a contributor to potent neutrophil recruitment during Gc infection.

We next sought to determine the source of LTB_4 released during Gc infection. While neutrophils are major producers of LTB_4, epithelial cells can potentially make this eicosanoid (Behera et al., 1998; Brock, 2005). However, expression of *alox5,* which encodes 5-LOX, was poorly detected in uninfected and infected End1 cells (Table 3). Pretreatment of End1 cells with the 5-lipoxygenase activity inhibitors, Zileuton or Caffeic acid did not affect neutrophil transepithelial migration following Gc infection (Figure 12C). Furthermore, stable lentiviral-mediated delivery of shRNA targeting *alox5* to End1 cells did not affect neutrophil migration (Figure 12D). Additionally, no LTB_4 was detected in the apical compartment of infected End1 monolayers in the absence of neutrophils (Figure 12A). Together these results indicate that End1 cells do not produce LTB_4 during Gc infection. Expression of BLT_1 by End1 cells was also negligible compared to the positive control of differentiated HL60 human promyelocytes, in keeping with reports that BLT_1 is primarily expressed in myeloid cells (Table 4) (Le Bel et al., 2014; Nemeth and Mocsai, 2016).

We then tested the hypothesis that neutrophils were the main source of LTB_4 in this system. In support of this hypothesis, Gc-induced transepithelial migration was abrogated in neutrophils that were pre-treated with Zileuton (Figure 13A). This was not due to general effects on neutrophil health or migratory ability, since Zileuton treatment did not inhibit neutrophil transepithelial migration towards fMLP. Since we had observed neutrophil migration to Gc as early as 75 minutes (Figure 8C), we investigated whether we could inhibit LTB_4-mediated amplification of Gc-induced neutrophil transepithelial migration over time by targeting BLT_1. When LY223982 added to the apical medium at 0, 60, or 75 minutes following basolateral addition of neutrophils, the number of apically migrated neutrophils was significantly reduced compared to the untreated condition (Figure 13B). This correlated with significantly less LTB_4 in the apical medium when LY223982 was added apically at 0 or 75 minutes, compared with no treatment (Figure 13C). We conclude that transepithelially migrated neutrophils produce LTB_4 over time to establish a chemotactic gradient for neutrophils.

2.3.3 *Gc infection stimulates endocervical cell signaling pathways leading to 12-lipoxygenase activation and consequent neutrophil transepithelial migration*

The observations above led us to hypothesize that infected endocervical cells produce a non-LTB_4 chemoattractant that coordinates neutrophil transmigration, which is then amplified by neutrophil-derived LTB_4. In support of this hypothesis, we identified a signaling pathway involving epithelial enzymes protein kinase C (PKC) and phospholipase A_2 (PLA_2) during neutrophil migration to Gc. Pre-treatment of End1 monolayers with the pan-PKC inhibitor chelerytherine chloride or the pan-PLA_2 inhibitor ONO-RS-081 completely inhibited neutrophil migration to Gc (Figure 14A-B). Neutrophil migration to Gc was sensitive to inhibition of cytosolic phospholipase A2 alpha ($cPLA_2\alpha$),

but not soluble PLA_2 (Figure 14B). Inhibition of PLA_2 in End1 cells reduced apical LTB_4 to uninfected, negative control concentrations (Figure 15), further supporting our hypothesis that transmigrated neutrophils are the major source for apical LTB_4.

IL-8 is elevated in acute gonorrhea, and is produced by End1 cells infected with Gc (Fichorova and Anderson, 1999; Fichorova et al., 2001; Fichorova et al., 1997). In agreement, we found that apically infected End1 monolayers released IL-8, with concentrations significantly higher in the basolateral than apical medium (Figure 14C). Addition of a neutralizing antibody against IL-8 to both the apical and basolateral medium had no effect on neutrophil migration to Gc, but inhibited migration across uninfected End1 cells to exogenous apical IL-8 (Figure 14D). Therefore IL-8, while produced by Gc-infected epithelial cells, is not required for basolateral-to-apical neutrophil migration in this system.

The requirement for $cPLA_2$ implied the involvement of epithelial-derived arachidonic acid (AA) and its metabolite(s) in neutrophil migration to Gc. In particular, AA-derived eicosanoids made by 12-lipoxygenase are neutrophil chemoattractants important for neutrophil influx to multiple mucosal bacterial pathogens (Pace-Asciak, 2015; Pazos et al., 2015; Szabady and McCormick, 2013). Pretreatment of End1 monolayers with the 12-lipoxygenase inhibitor cinnamyl-3,4-dihydroxy-α-cyanocinnamate completely inhibited Gc-dependent neutrophil transepithelial migration (Figure 15A). AA can also be converted to prostaglandins via COX-2, however, pre-treatment of End1 monolayers with the COX-2 inhibitor NS398 had no effect on neutrophil transepithelial migration to Gc (Figure 17). We therefore focused on 12-lipoxygenases downstream of PKC and PLA_2 in epithelial cells.

12-lipoxygenase activity in humans can be catalyzed by 15-LOX, 12-LOX, and 12R-LOX/eLOX-3, encoded by *alox15, alox12, alox12b, and aloxe3* respectively {Tamang, 2012 #275;Kuhn, 2015 #495;Kuhn, 2002 #760}. Of these, only *alox12b* and

aloxe3 were expressed in End1 cells (Table 3), with expression significantly increasing following Gc infection (Figure 16B). Unlike other 12-lipoxygenases, 12R-LOX is reported to work in concert with eLOX-3 to produce hepoxilin A_3 (HXA_3) (Epp et al., 2007; Krieg et al., 2013; Mashima and Okuyama, 2015; Yu et al., 2003). Stable shRNA knockdown of *alox12b* or *aloxe3* (Figure 16B) significantly decreased neutrophil migration to Gc (Figure 16C), without affecting migration to apically added LTB_4 (data not shown). These results implicate 12R-LOX and eLOX-3 in coordinating the endocervical response to Gc resulting in neutrophil transepithelial migration.

Three lines of evidence suggested HXA_3 was the 12-lipoxygenase product required for endocervical cells to support neutrophil migration. First, apical addition of recombinant sEH, which hydrolyzes the epoxide ring of HXA_3 required for neutrophil chemoattraction to yield the inert trioxilin A_3 (TrA_3) (Cronin et al., 2011; Morisseau and Hammock, 2013; Morisseau et al., 2012; Pace-Asciak, 2015), significantly inhibited neutrophil migration to Gc (Figure 16D). sEH addition did not affect neutrophil migration to an imposed apical gradient of LTB_4 (Figure 16D), which does not contain an epoxide ring and is thus insensitive to sEH. Second, HXA_3 is secreted apically by MRP2 channels in the context of other mucosal bacterial infections (Pazos et al., 2008). Inhibition of the MRP2 efflux pump on End1 cells with MK571 significantly inhibited neutrophil migration to Gc (Figure 16D). Third, lipid-extracted supernatants from infected End1 monolayers stimulated 2-5 fold more neutrophil migration across an acellular insert compared to mock-infected supernatants, a statistically significant increase (Figure 18). HXA_3 is known to partition into the lipid fraction of infection supernatants (Pazos et al., 2015; Tamang et al., 2012). Taken together, we conclude that endocervical cells infected with Gc stimulate 12R-LOX and eLOX-3 lipoxygenase activity to produce an epoxide-containing eicosanoid, likely HXA_3, which is essential for neutrophil transepithelial migration.

2.3.4 *Neutrophil migration to Gc-endocervical cell infection involves a trypsin-sensitive Gc surface factor(s)*

Since neutrophil migration required Gc-endocervical cell contact (Figure 8), we examined surface features of Gc that might be responsible. Using isogenic derivatives constitutively expressing type IV pili and/or opacity-associated (Opa) proteins, two major Gc adhesins (Ball and Criss, 2013), there was no difference in neutrophil migration when normalized to equivalent adhered CFU (Figure 19A, Table 2). We took a less biased approach by measuring neutrophil migration to Gc gently treated with trypsin. When compared with vehicle-treated Gc, we measured significantly less neutrophil migration to trypsin-treated Gc (Figure 19B). Trypsin treatment did not affect Gc viability (data not shown) or adherence to End1 monolayers (Figure 19C). These results implicate one or more trypsin-sensitive Gc surface components in initiating epithelial coordination of neutrophil migration during infection.

2.4 Discussion

Gc infection leads to sustained neutrophil influx, but the mechanisms underlying recruitment of neutrophils across the epithelium in the context of Gc infection were not previously understood. Our three-component *in vitro* system demonstrates that Gc-endocervical cell contact stimulates eicosanoid-driven neutrophil transepithelial migration (Figure 20). Following Gc infection, endocervical cells produced an apically directed 12-lipoxygenase-derived neutrophil chemoattractant (HXA_3) that required efflux through MRP2 and was sensitive to sEH. This pathway was dependent on epithelial PKC and $cPLA_2$ to produce the12-lipoxygenase substrate AA. Transmigrated neutrophils

produced the 5-lipoxygenase product LTB_4, which amplified the apical-to-basolateral neutrophil chemotactic gradient. To our knowledge, this is the first time eicosanoids have been implicated in neutrophil influx to Gc.

The LTB_4-BLT_1 axis is important for directing neutrophil chemotaxis to sites of inflammation (Nemeth and Mocsai, 2016). Our results reveal a role for LTB_4 in neutrophil influx to Gc. *In vivo*, multiple additional cell types and factors likely influence the inflammatory milieu during Gc infection. For example, Gc skews the immune response away from a protective adaptive immune response and towards a Th17-driven neutrophilic response during human infection (Stevens and Criss, 2018). Interestingly, LTB_4 has recently been shown to induce Th17 cell migration to a greater extent than Th1 or Th2 cell migration (Lee et al., 2015). Neutrophil migration and LTB_4 signaling activate neutrophils to combat invading pathogens (Le Bel et al., 2014; Nadeau et al., 2002). Neutrophils recruited to Gc infection appeared protrusive, though their activation status remains to be determined. However, given that Gc can evade neutrophil killing, neutrophil activation may not control infection and instead contribute to host cell damage.

Our results indicate a role for epithelial 12R-LOX and eLOX-3 during neutrophil migration to Gc. This was surprising, as other bacterial pathogens studied to date stimulate 15-LOX to produce HXA_3 (Boll et al., 2012a; Mumy et al., 2008). 12R-LOX is primarily expressed in skin and is unique in that it requires downstream activity of eLOX-3 to generate HXA_3 (Epp et al., 2007; Krieg et al., 2013; Mashima and Okuyama, 2015; Yu et al., 2003). The best described function of 12R-LOX and eLOX-3 is to prevent cellular water loss, with mutations in both linked to dry skin disorders (Epp et al., 2007; Krieg et al., 2013; Mashima and Okuyama, 2015). Interestingly, the first reports of 12R-LOX in the context of disease describe accumulation of its product 12R-HETE in psoriatic scales (Boeglin et al., 1998), suggesting that 12R-LOX plays a role during

certain inflammatory states. eLOX3 is described as a hepoxilin synthase, and our combined evidence with sEH, the MRP2 efflux pump that exports HXA_3, and neutrophil chemotactic activity in lipid-enriched fractions from infected End1 supernatants all suggest that HXA_3 coordinates this event. HXA_3 is a highly potent yet labile chemoattractant, which has complicated confirmation of this eicosanoid in this *in vitro* system.

Many laboratories have demonstrated that pathogens stimulate eicosanoid signaling by affecting AA production (Bhowmick et al., 2013; Hurley et al., 2004; McCormick et al., 1995; Mrsny et al., 2004; Mumy et al., 2008; Pazos et al., 2015; Szabady and McCormick, 2013). In agreement, we found that endocervical PKC and PLA_2 are required for neutrophil migration to Gc. Similar to what we observed during Gc-endocervical cell infection, *S. flexneri* and *S. pneumoniae* specifically activate epithelial $cPLA_2\alpha$, which is required for neutrophil migration (Bhowmick et al., 2017; Mumy et al., 2008), However, Gc does not possess the secreted or surface factors employed by these mucosal pathogens to stimulate eicosanoid production (Pazos et al., 2017; Silva et al., 2004; Zurawski et al., 2006). Instead, our study implicates (a) trypsin-sensitive Gc surface factor(s) in stimulating contact-dependent neutrophil migration. Gc also differs from other mucosal bacterial pathogens in surface features it uses to adhere to and interact with host cells. Two major Gc adherence factors, Opa proteins and type IV pili, were not required for neutrophil migration. Therefore, the Gc factors that stimulate eicosanoid production will be an important topic for future investigation.

Broadly, our findings highlight the importance of studying context-specific bacterial-epithelial interactions that engage eicosanoid-producing pathways in order to identify novel therapeutic targets for infections in which disease is exacerbated by neutrophilic inflammation. Specifically for gonorrhea, sustained neutrophil influx that does not resolve Gc infection contributes to host cell damage. With increased failures of

antibiotic treatment, we anticipate increased likelihood for inflammatory damage, especially in women, leading to PID, ectopic pregnancy, and infertility. Our work suggests targeting this eicosanoid pathway at the cervix would be an attractive adjunctive therapeutic strategy to mitigate severe clinical sequelae in infected women. As precedence, immunosuppressive therapy is added to treatment regimens for bacterial meningitis and Pneumocystis pneumonia in patients with HIV (Barichello et al., 2015; Ewald et al., 2015). There is also precedence for targeting lipoxygenase pathways in inflammation, with FDA-approved therapeutics for asthma including Zileuton and the leukotriene receptor antagonist Monteleukast. This study reveals novel lines of inquiry into gonorrheal disease pathogenesis in order to ameliorate the negative, lifelong consequences of gonorrhea in women.

2.5 Figures for Chapter 2

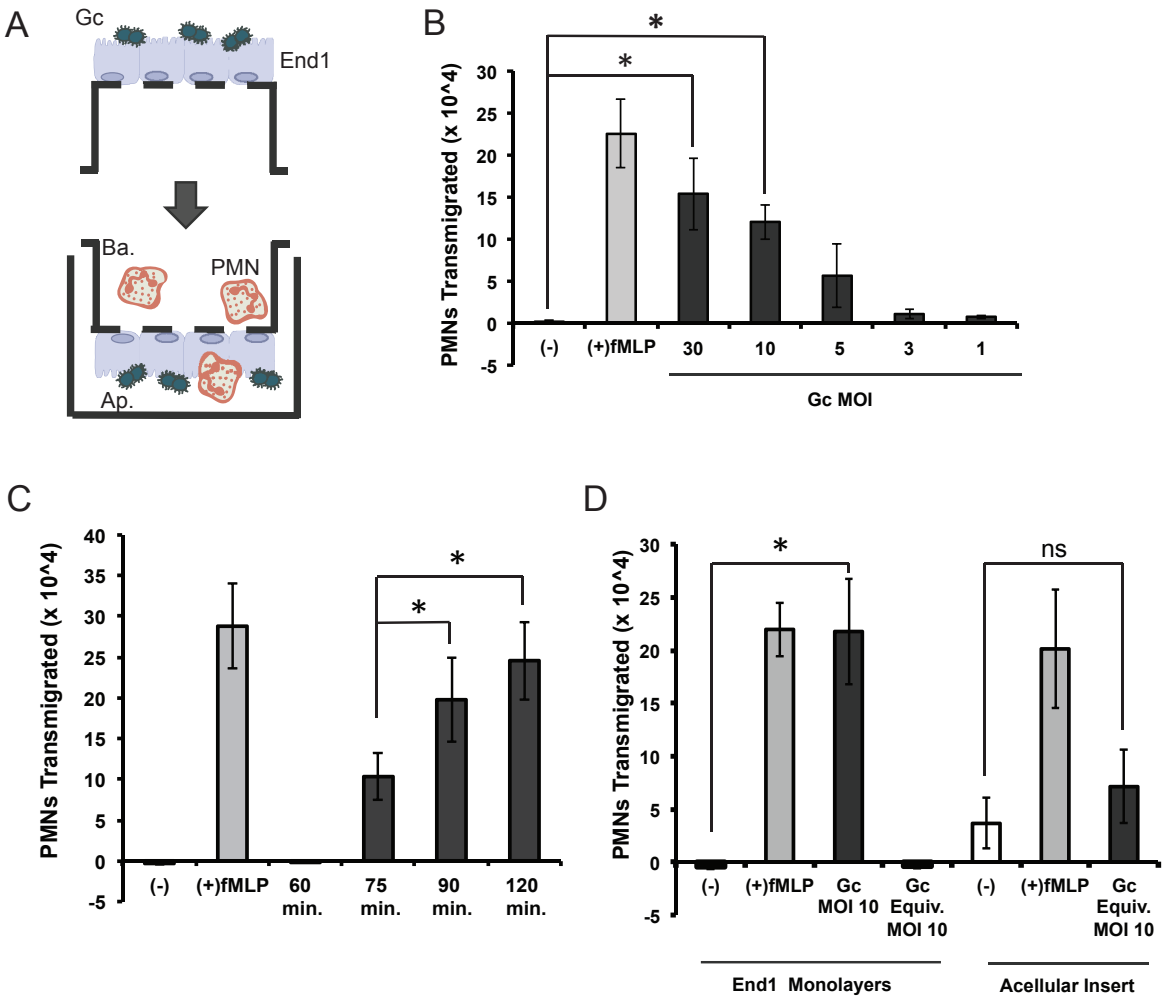


Figure 8. Contact-dependent *N. gonorrhoeae* infection of polarized endocervical cell monolayers stimulates neutrophil transepithelial migration. End1 monolayers were grown on the underside of collagen-coated Transwell™ inserts (6.5 mm diameter, 3.0 μm pores). Neutrophil transepithelial migration was quantified by myeloperoxidase (MPO) assay. **(A)** Schematic of in vitro three-component system depicting apical infection and neutrophil transepithelial migration. Polarized End1 monolayers were inverted in a humidified chamber and apically infected with Gc. Following infection, infected monolayers were thoroughly washed in $HBSS^{+}$ to remove non-adherent bacteria and reverted into a 24-well tissue culture well containing 1 mL $HBSS^{+}$. Primary human neutrophils (1 x 10^6) were added to the basolateral reservoir and transmigration

allowed to proceed for 2 hr unless otherwise noted. Monolayers are then removed and the number of neutrophils in the apical reservoir quantified by myeloperoxidase (MPO) assay. **(B)** Polarized End1 monolayers were infected at various MOIs for 1 hr and then assayed for neutrophil transepithelial migration. **(C)** Polarized End1 monolayers were apically infected with Gc at an MOI=10 for 1 hr, and then neutrophil transmigration assayed after 60, 75, 90, and 120 minutes. **(D)** End1 monolayers were apically infected with Gc at MOI=10, an equivalent number of Gc was added to the apical reservoir without interacting with End1 cells, or an equivalent number of Gc was added to the apical reservoir an acellular Transwell^{TM} insert and incubated for 1 hr before assaying for neutrophil transmigration. **(B-D)** White bars represent neutrophil transepithelial migration to buffer alone with no apical stimulus, (-), grey bars represent neutrophil transepithelial migration to an imposed apical gradient of *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine, (+)fMLP, and black bars represent neutrophil migration to Gc. Results are expressed as a mean ± standard error of the mean for at least three independent experiments per condition. Statistics for **(B)** were calculated using a One-way ANOVA with Tukey post-hoc test and statistics for **(C)** and **(D)** were calculated using a two-tailed, unpaired Student's *t*-test. $*P < 0.05$. Abbreviations: Gc, *Neisseria gonorrhoeae*; ns, not significant; MOI, multiplicity of infection; End1, End1 E6/E7 cells; Ap., apical; Ba., basolateral; PMN, polymorphonuclear cell/neutrophil.

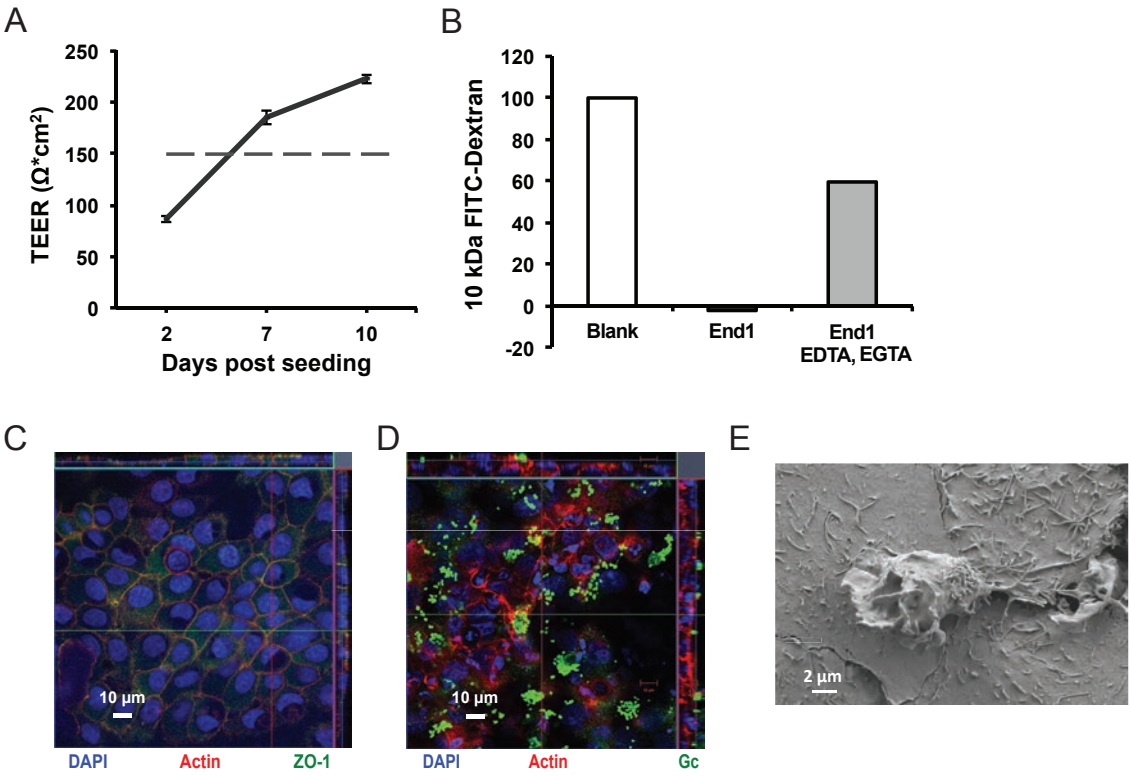


Figure 9. End1 cells from polarized monolayers on inverted Transwell™ inserts that support apical infection with Gc and neutrophil transepithelial migration. End1 monolayers were grown on the underside of collagen-coated Transwell™ inserts (6.5 mm diameter, 3.0 µm pores). **(A)** Transepithelial electrical resistance (TEER) over time. Mature monolayers achieve TEER ≥ 150 Ω • cm2. **(B)** Paracellular flux of 10 kDa FITC-dextran across End1 monolayers (black bar) compared to monolayers treated with 10 mM EDTA and 10 mM EGTA (grey bar) or acellular inserts (white bar). **(C)** Visualization of ZO-1 staining of a 10-day-old End1 monolayer. ZO-1 (green), actin (red), and DAPI (blue). The confocal image was captured on a Zeiss LSM-700. **(D)** Visualization of Gc-infected End1 monolayer. Gc (green), actin (red), and DAPI (blue). The confocal image was captured on a Zeiss LSM-700. **(E)** Following apical infection with Gc at an MOI=10 and neutrophil transepithelial migration, End1 monolayers were fixed in 4% paraformaldehyde/2% glutaraldehyde and processed for SEM by the advanced microscopy facility at UVA. SEM images were captured on a Zeiss SEM.

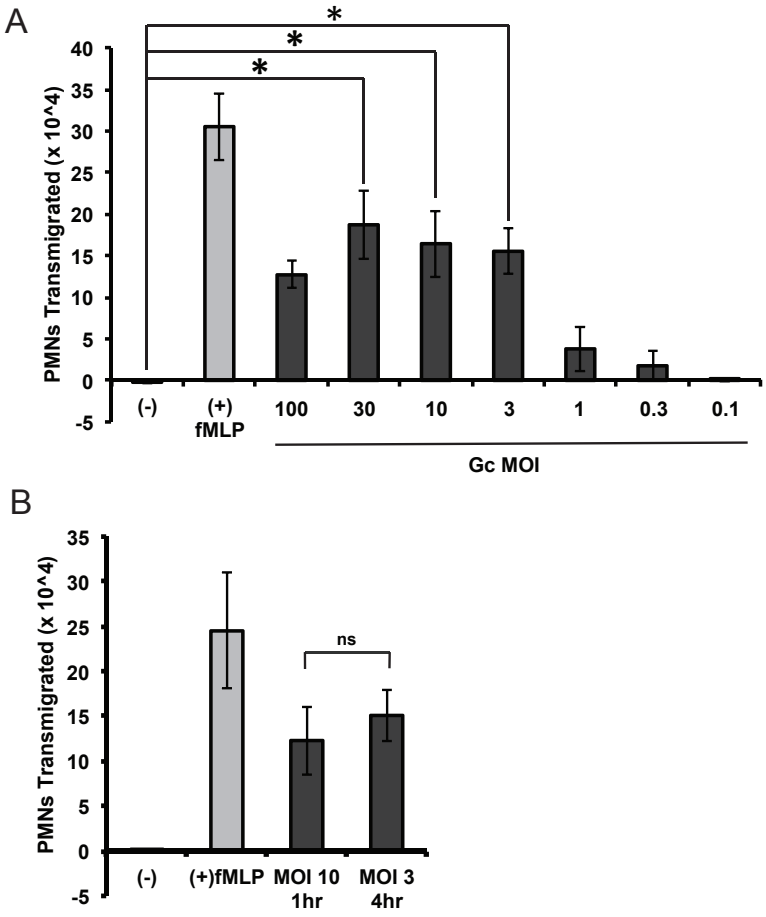


Figure 10. Neutrophils migrate to both low and high Gc inoculum over time. White bars represent neutrophil transepithelial migration to buffer alone with no apical stimulus, (-), grey bars represent neutrophil transepithelial migration to an imposed apical gradient of *N*-formyl-L-methionl-L-leucyl-L-phenylalanine, (+)fMLP, and black bars represent neutrophil migration to Gc. **(A)** Polarized End1 monolayers were infected at various MOIs for 4 hr and then assayed for neutrophil transepithelial migration. **(B)** Polarized End1 monolayers were infected with Gc for one hour at an MOI=10 or for 4 hr at an MOI=3 and then assayed for neutrophil transepithelial migration. Results are expressed as a mean ± standard error of the mean for at least three independent experiments per condition. Statistics for **(A)** were calculated using a One-way ANOVA with Tukey post-hoc test. Statistics for **(B)** were calculated using a two-tailed, unpaired Student's *t*-test.

*$P < 0.05$. Abbreviations: Gc, *Neisseria gonorrhoeae*; ns, not significant; MOI, multiplicity of infection; PMN, polymorphonuclear cell/neutrophil.

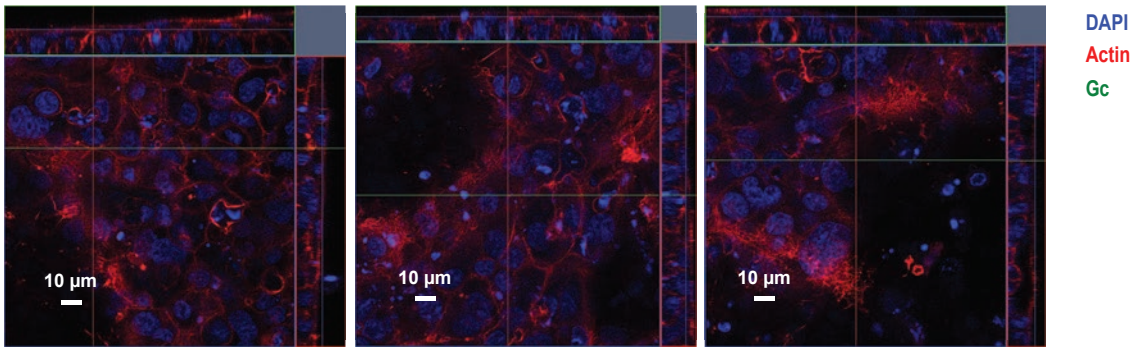


Figure 11. End1 monolayers with Gc added to the apical reservoir do not exhibit adhered Gc. Representative confocal microscopy images of End1 monolayers. Gc was added to the apical reservoir of the tissue culture well for 1 hr. End1 monolayers on inverted Transwell^{TM} inserts were added to the well immediately prior to assaying for neutrophil migration. Following neutrophil migration, End1 monolayers were stained for adhered Gc. Gc (green), actin (red), and DAPI (blue). The confocal image was captured on a Zeiss LSM-700. Abbreviations: Gc, *Neisseria gonorrhoeae.*

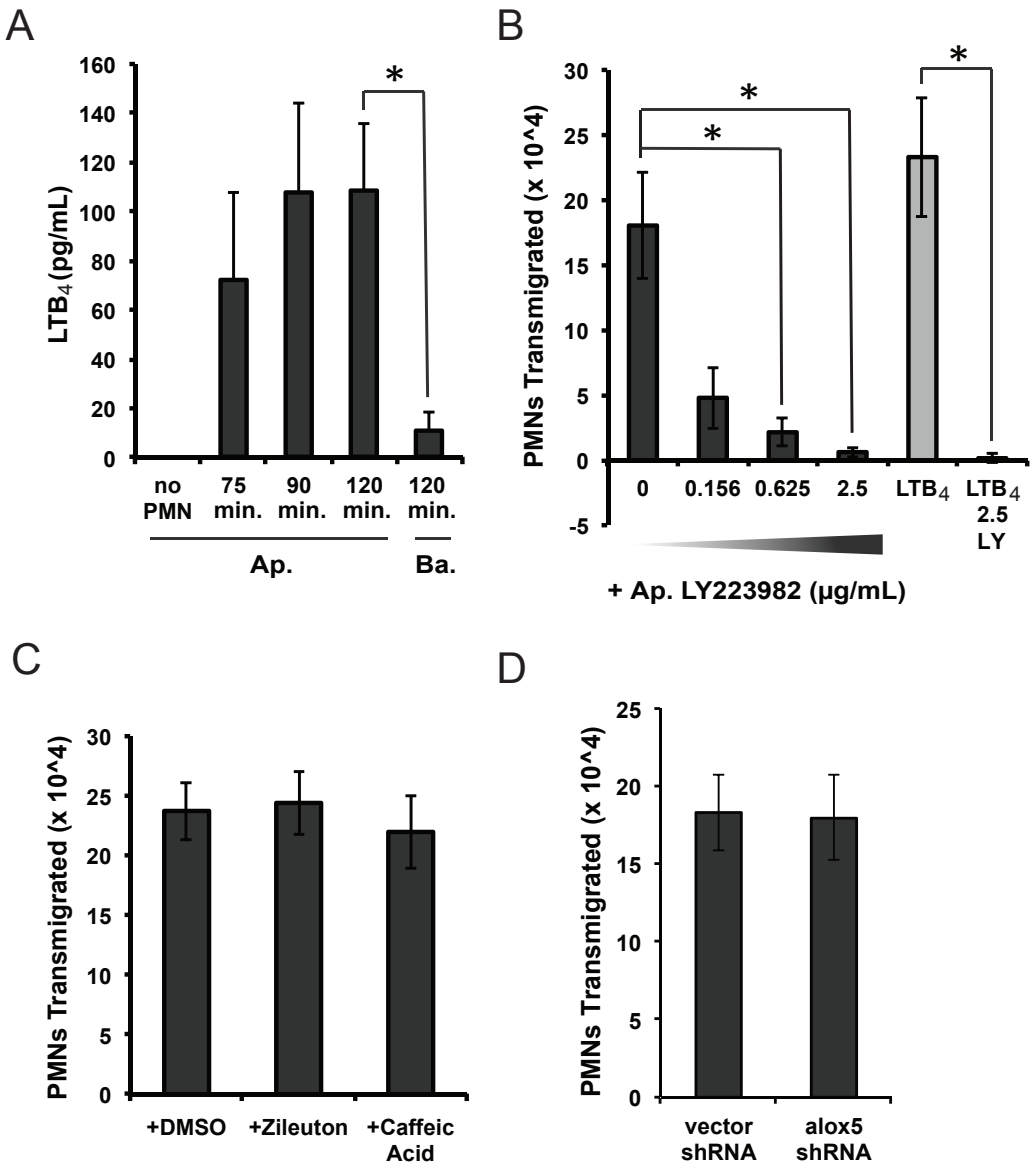


Figure 12. Neutrophil migration to *N. gonorrhoeae* requires leukotriene B_4 but not epithelial 5-lipoxygenase activity. (A) Supernatants from apical and basolateral compartments following Gc infection and/or neutrophil transepithelial migration were collected at the indicated time points and conditions, passed through a 0.2 µm filter, and LTB_4 quantified by enzyme-linked immunosorbent assay (ELISA). **(B)** Polarized End1 monolayers were infected with Gc at an MOI=10 for 1 hr. During neutrophil transepithelial migration, LY223982, an antagonist to the high affinity LTB_4 receptor

BLT_1, was added to the apical reservoir immediately prior to addition of neutrophils at the indicated concentrations (μg/mL). Grey bars represent neutrophil transepithelial migration to an imposed apical gradient of LTB_4 and black bars represent neutrophil migration to Gc. **(C)** Polarized End1 monolayers were pre-treated with 5-lipoxygenase inhibitor Zileuton (25 μM), Caffeic acid (50 μM), or an equivalent concentration of the vehicle control (DMSO) diluted in $HBSS^+$ for 1 hr prior to Gc infection, thoroughly washed, and infected at an MOI=10 and neutrophil transepithelial migration. There was no significant difference in neutrophil migration. **(D)** Neutrophil transepithelial migration was assayed across End1 monolayers infected with Gc at an MOI=10 for 1 hr, with End1 cells either stably transformed with vector shRNA or shRNA against *alox5*. For all panels, results are expressed as a mean ± standard error of the mean for at least three independent experiments per condition. Statistics for were calculated using a two-tailed, **(A)** paired or **(B)** unpaired Student's *t*-test. $*P < 0.05$. Abbreviations: Gc, *Neisseria gonorrhoeae*; End1, End1 E6/E7 cells; Ap., apical; Ba., basolateral; PMN, polymorphonuclear cell/neutrophil; LTB_4, leukotriene B_4.

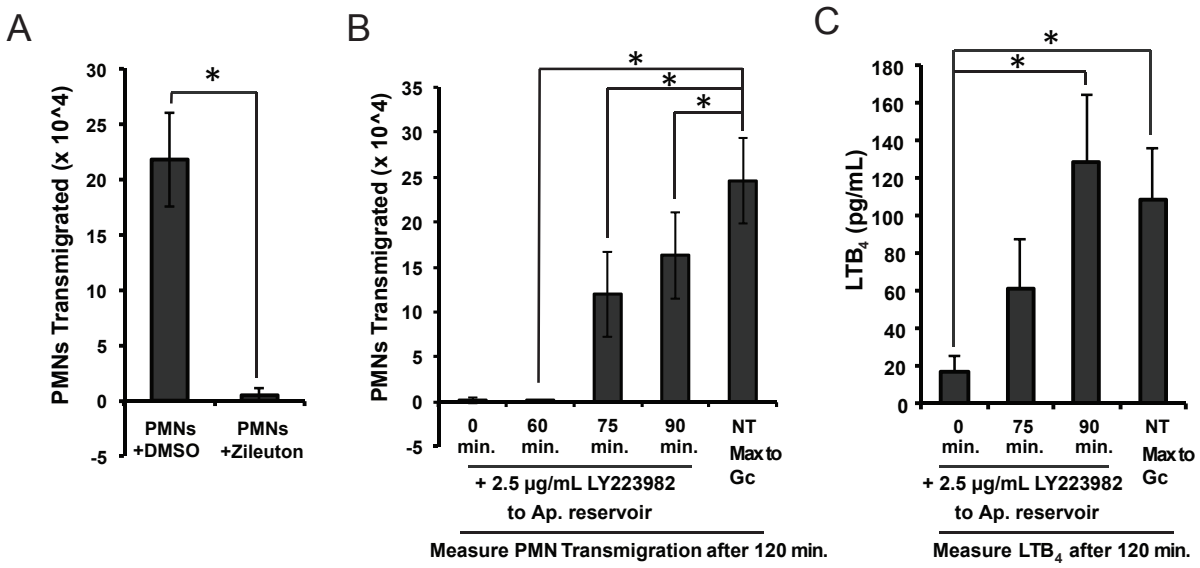


Figure 13. Neutrophils produce leukotriene B_4 to amplify neutrophil transepithelial migration to *N. gonorrhoeae*. (A) Primary human neutrophils were pre-treated with the 5-lipoxygenase inhibitor Zileuton (25 µM) or vehicle (DMSO) prior to assaying neutrophil transepithelial migration across polarized End1 monolayers infected with Gc at an MOI=10 for 1 hr. **(B)** LY223982 (2.5 µg/mL), an antagonist to the high affinity LTB_4 receptor BLT_1, was added to the apical reservoir of infected End1 monolayers at the indicated time points during neutrophil transmigration (0, 60, 75, and 90 minutes). Neutrophil transmigration after addition of LY223982 is compared to the maximum migration seen to apical Gc infection after 120 minutes with no treatment (NT). **(C)** Supernatants from apical and basolateral compartments following Gc infection and/or neutrophil transepithelial migration were collected at the indicated time points and conditions, passed through a 0.2 µm filter, and LTB_4 quantified by ELISA. Results are expressed as a mean ± standard error of the mean for at least three independent experiments per condition. Statistics for were calculated using a two-tailed, **(A-B)** unpaired or **(C)** paired Student's *t*-test. *P < 0.05. Abbreviations: Gc, *Neisseria*

gonorrhoeae; MOI, multiplicity of infection; PMN, polymorphonuclear cell/neutrophil; Ap., apical; LTB_4, leukotriene B_4.

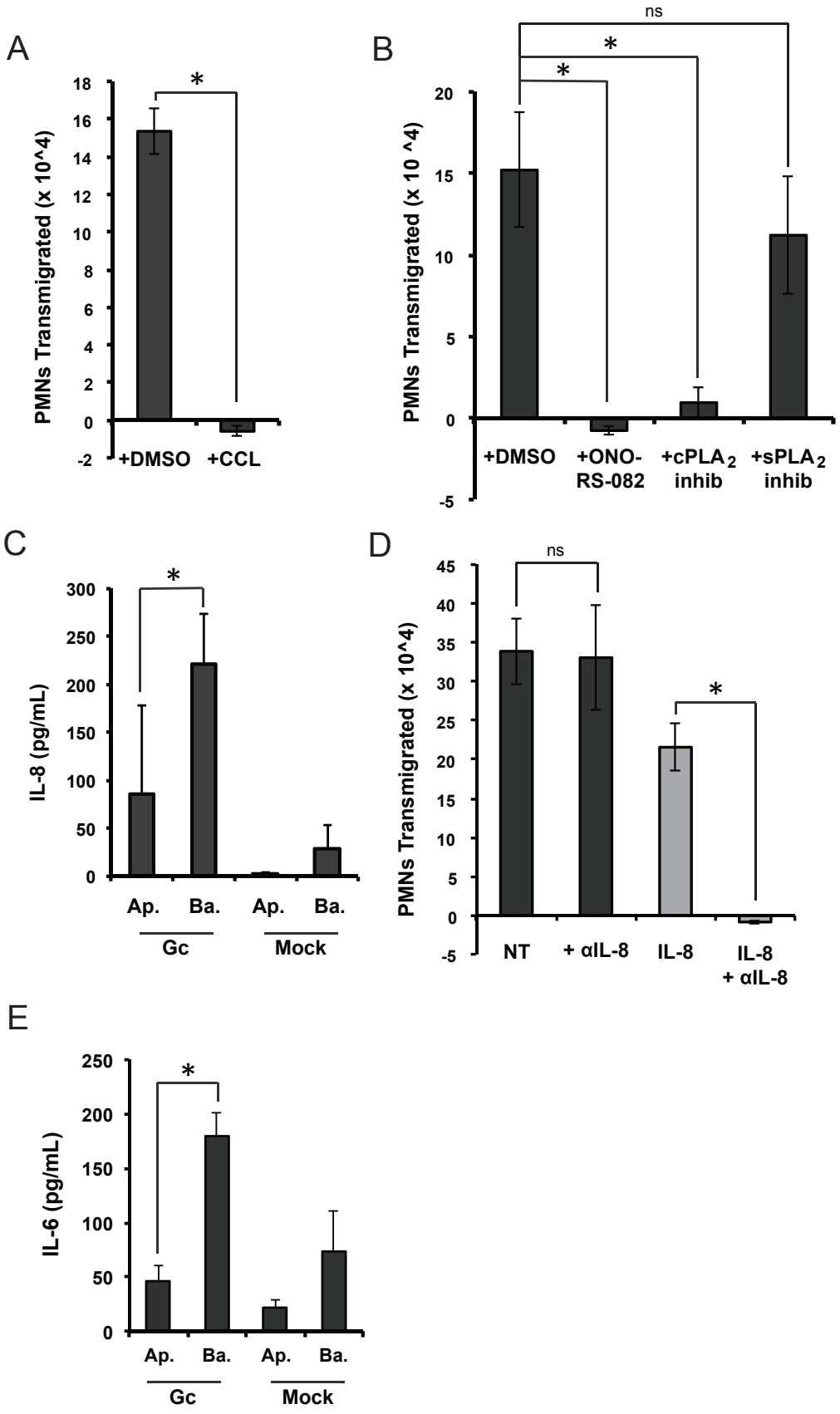

A
PMNs Transmigrated (x 10^4)
18
16
14
12
10
8
6
4
2
0
-2
*
+DMSO
+CCL
B
PMNs Transmigrated (x 10 ^4)
20
15
10
5
0
-5
ns
*
*
+DMSO
+ONO-RS-082
+cPLA2 inhib
+sPLA2 inhib
C
IL-8 (pg/mL)
300
250
200
150
100
50
0
*
Ap.
Ba.
Ap.
Ba.
Gc
Mock
D
PMNs Transmigrated (x 10^4)
45
40
35
30
25
20
15
10
5
0
-5
ns
*
NT
+ αIL-8
IL-8
IL-8 + αIL-8
E
IL-6 (pg/mL)
250
200
150
100
50
0
*
Ap.
Ba.
Ap.
Ba.
Gc
Mock

Figure 14. Neutrophil transepithelial migration to *N. gonorrhoeae* requires an epithelial pathway involving protein kinase C and phospholipase A_2, but not apically directed interleukin-8. For treatment with inhibitors of epithelial cell targets, epithelial cells were pre-treated with the inhibitor and then washed thoroughly before infection with Gc at an MOI=10 and neutrophil transepithelial migration. **(A)** Polarized End1 monolayers were pre-treated with the pan-PKC inhibitor chelerythrine chloride (CCL) (5 μM), or **(B)** the pan-PLA_2 inhibitor ONO-RS-081 (5 μM), cPLA_2α-inhibitor (6 μM), or sPLA_2-inhibitor 2,4'-Dibromoacetophenone (7 μM). **(C)** Supernatants from apical and basolateral compartments following a 3 hr Gc infection or mock infection were collected and passed through a 0.2 μm filter, and IL-8 quantified by ELISA. **(D)** Polarized End1 monolayers were infected with Gc at an MOI=10 for 1 hr. During neutrophil transepithelial migration, an IL-8 blocking antibody (20 μg/mL), was added to the apical and basolateral reservoirs immediately prior to addition of neutrophils. Grey bars represent neutrophil transepithelial migration to an imposed apical gradient of IL-8, and black bars represent neutrophil migration to Gc. Results are expressed as a mean ± standard error of the mean for at least three independent experiments per condition. Statistics were calculated using a two-tailed, **(A), (B),** and **(D)** unpaired or **(C)** paired Student's *t*-test. **P* < 0.05. Abbreviations: Gc, *Neisseria gonorrhoeae*; ns, not significant; Ap., apical; Ba., basolateral; PMN, polymorphonuclear cell/neutrophil; IL-8, interleukin-8.

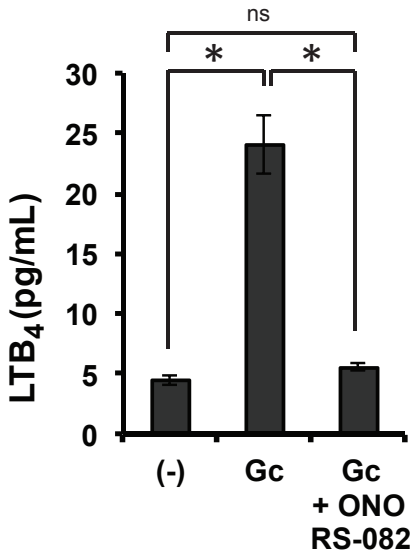


Figure 15. Inhibition of neutrophil migration by inhibiting End1 PLA_2 activity inhibits apical LTB_4 production. End1 monolayers were pretreated with the pan-PLA_2 inhibitor ONO-RS-082 (ONO, 5 μM) for 1 hr prior to infection and neutrophil transmigration as in Fig. 1D. Supernatants from apical compartments following Gc infection and neutrophil transepithelial migration were collected following neutrophil transmigration, passed through a 0.2 μm filter, and LTB_4 quantified by ELISA. Results are expressed as a mean ± standard error of the mean for at least three independent experiments per condition. Statistics were calculated using a two-tailed paired Student's *t*-test. **P* < 0.05. Abbreviations: Gc, *Neisseria gonorrhoeae*; ns, not significant; LTB_4, leukotriene B_4.

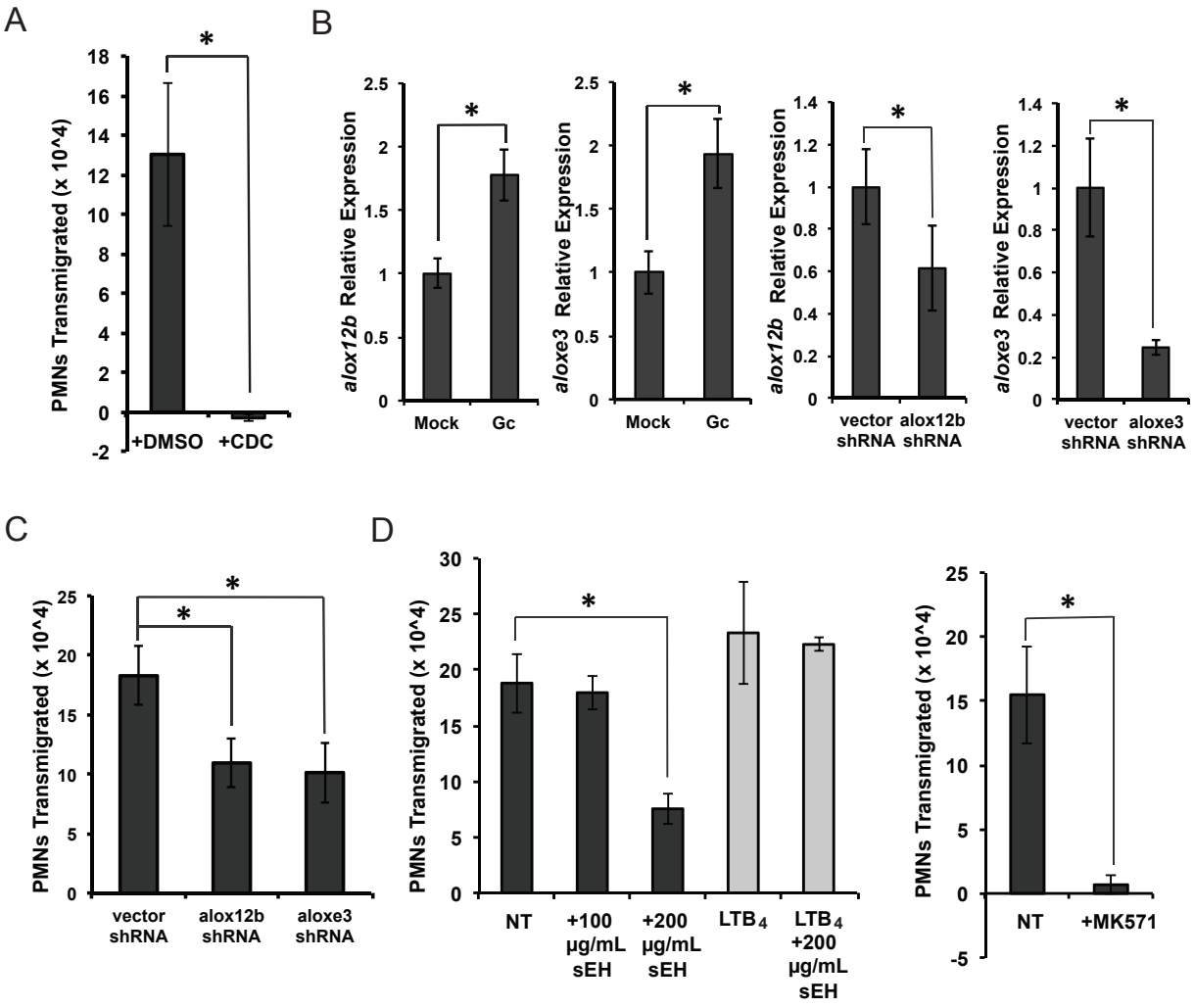


Figure 16. Neutrophil transepithelial migration requires an epithelial 12-lipoxygenase-dependent chemoattractant that is sensitive to soluble epoxide hydrolase and is secreted through the MRP2 channel. (A) Polarized End1 monolayers were pre-treated with 12-lipoxygenase inhibitor Cinnamyl -3,4-dihydroxy-α-cyanocinnamate (CDC) (50 µM) or an equivalent concentration of the vehicle control (DMSO) for 1 hr, thoroughly washed, infected at an MOI=10, and assayed for neutrophil transepithelial migration. **(B)** Relative expression of *alox12b* or *aloxe3* in End1 monolayers either mock infected or infected with Gc at an MOI=10 for 3 hr or in End1

monolayers following lentiviral-mediated shRNA knockdown of *alox12b* or *aloxe3* or vector control. **(C)** Neutrophil transepithelial migration was assayed across End1 monolayers infected with Gc at an MOI=10 for 1 hr, with End1 cells either stably transformed with vector shRNA or shRNA against *alox12b* or *aloxe3*. **(D)** Neutrophil transepithelial migration was assayed across End1 monolayers infected with Gc at an MOI=10 for 1 hr, with either 100 μg/mL or 200 μg/mL human recombinant soluble epoxide hydrolase (sEH) added to the apical reservoir prior to and throughout neutrophil transepithelial migration or pre-treatment of polarized End1 monolayers with MRP-2 inhibitor MK571 (100 μM). **(A, C-D)** Grey bars represent neutrophil transepithelial migration to an imposed apical gradient of LTB_4 and black bars represent neutrophil migration to Gc. Results are expressed as a mean ± standard error of the mean for at least three independent experiments per condition. Statistics for were calculated using a two-tailed, unpaired Student's *t*-test. **P* < 0.05. Abbreviations: Gc, *Neisseria gonorrhoeae*; ns, not significant; NT, no treatment; Ap., apical; Ba., basolateral; PMN, polymorphonuclear cell/neutrophil; LTB_4, leukotriene B_4; sEH, soluble epoxide hydrolase.

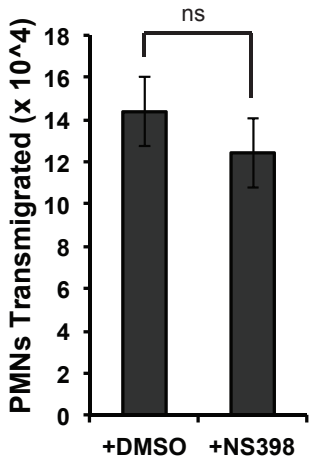


Figure 17. Neutrophil transmigration to Gc does not require End1 cyclooxygenase-2 activity. Polarized End1 monolayers were pre-treated with cyclooxygenase-2 specific inhibitor NS398 (50 μM) or an equivalent concentration of the vehicle control (DMSO) for 1 hr then thoroughly washed prior to Gc infection at an MOI=10 and neutrophil transepithelial migration. Results are expressed as a mean ± standard error of the mean for at least three independent experiments per condition. Statistics for were calculated using a two-tailed, unpaired Student's *t*-test. **P* < 0.05. Abbreviations: ns, not significant; PMN, polymorphonuclear cell/neutrophil.

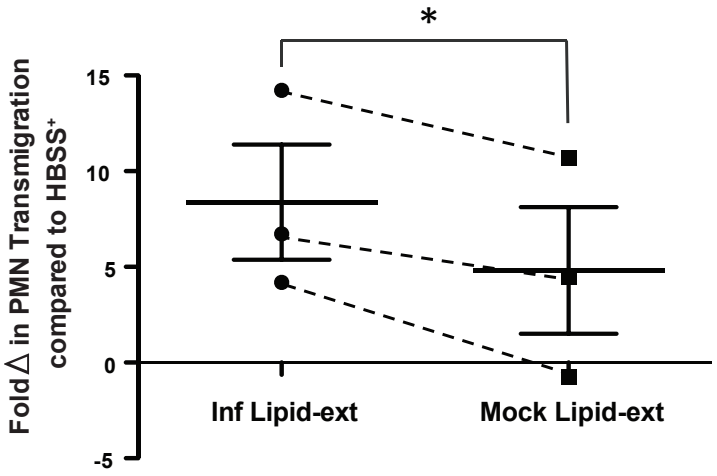


Figure 18. The endocervical cell-derived neutrophil chemoattractant is in the lipid-extracted supernatant of Gc-infected End1 monolayers. Non-polarized End1 cells in 162-cm^2 flasks were infected at an MOI=100 for 1hr. Supernatants were collected, lipid-extracted, and added to the apical reservoir of an acellular Transwell^{TM} insert. Results are expressed as a mean ± standard error of the mean for at least three independent experiments per condition, with paired experiments indicated by dotted lines. Statistics were calculated using a two-tailed, paired Student's *t*-test. *P < 0.05. Abbreviations: PMN, polymorphonuclear cell; Inf, infected; HBSS$^+$, modified hank's basic salt solution.

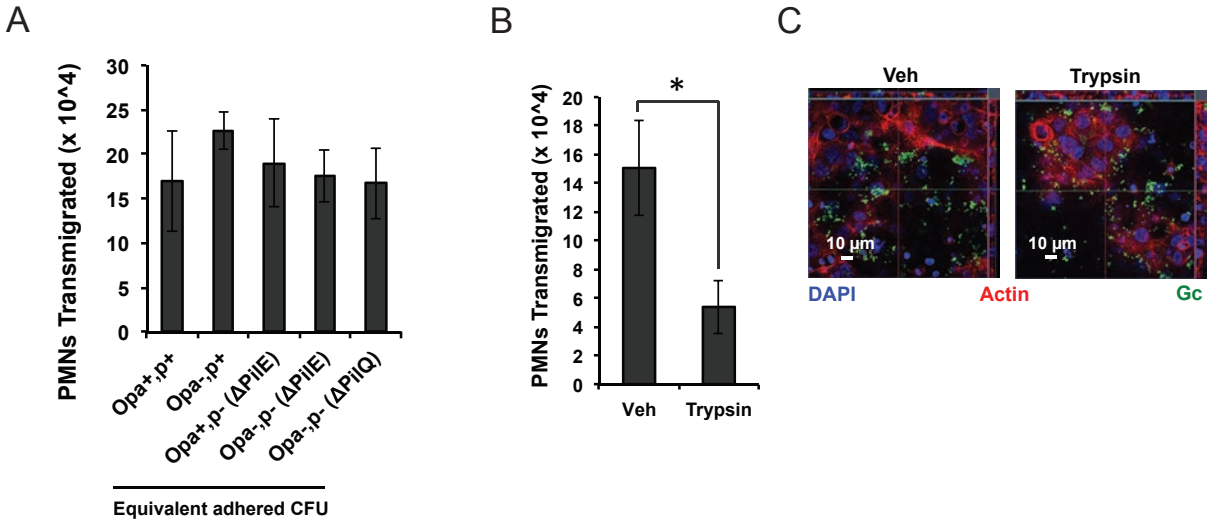


Figure 19. Neutrophil transmigration to *N. gonorrhoeae* involves trypsin-sensitive bacterial surface factor(s). (A) Polarized End1 monolayers were infected with isogenic derivatives of strain of FA1090 Gc at an MOI=10-20 (to normalize Gc-End1 association after 1hr). **(B)** Neutrophil transmigration was assayed to trypsin- or vehicle-treated Gc. **(C)** Representative images of End1 monolayers infected with trypsin- or vehicle-treated Gc. Gc (green), actin (red), and DAPI (blue). The confocal image was captured on a Zeiss LSM-700. Results are expressed as a mean ± standard error of the mean for at least three independent experiments per condition. Statistics for were calculated using a two-tailed, unpaired Student's *t*-test. **P* < 0.05. Abbreviations: Gc, *Neisseria gonorrhoeae*; Opa, opacity-associated protein; p, type IV pilus; ns, not significant; MOI, multiplicity of infection; End1, End1 E6/E7 cells; Veh, vehicle; PMN, polymorphonuclear cell/neutrophil.

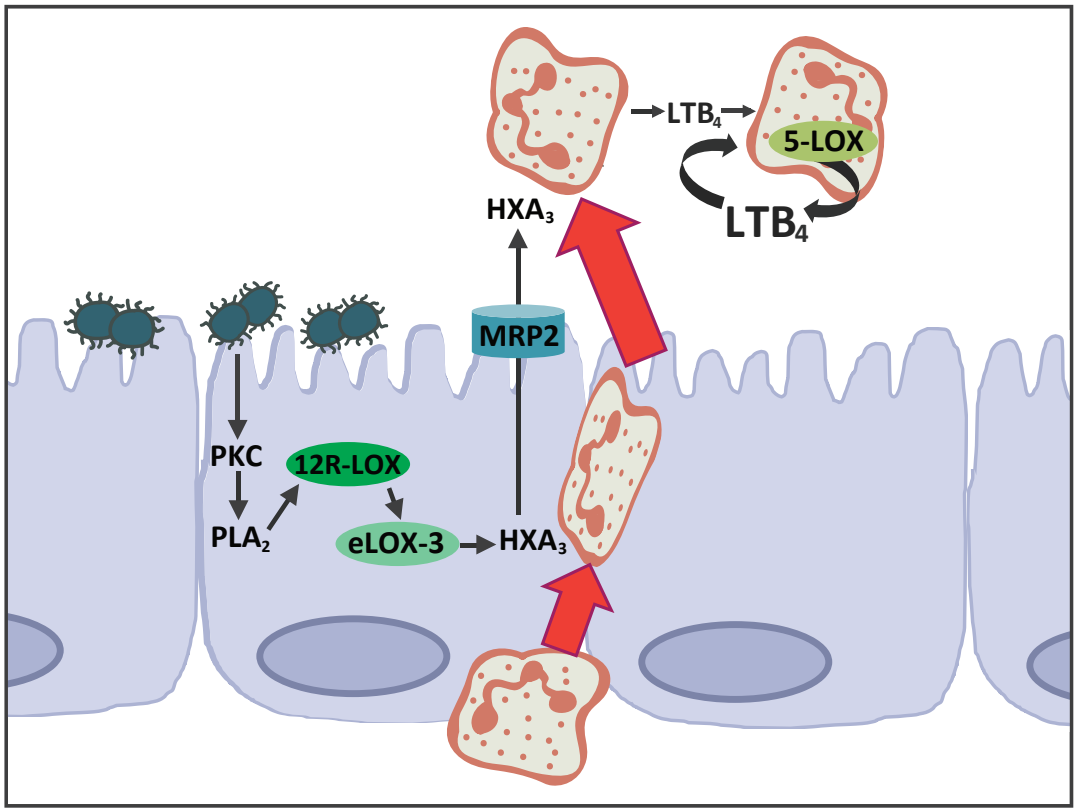


Figure 20. Model of eicosanoid-coordinated neutrophil migration across *N. gonorrhoeae*-infected endocervical cells. Gc infection stimulates End1 protein kinase C (PKC), phospholipase A_2 (PLA_2), and lipoxygenases (LOX) 12R-LOX and eLOX-3 to produce the eicosanoid hepoxilin A_3 (HXA_3) that is directed apically by MRP2 channels. Sentinel transmigrating neutrophils activate 5-LOX to produce LTB_4 that acts in paracrine and autocrine fashion to amplify neutrophil influx. Abbreviations: End1, End1 E6/E7 cells; PKC, protein kinase C; PLA_2, phospholipase A_2, 12R-LOX, arachidonate 12-lipoxygenase; eLOX-3, epidermis-type lipoxygenase-3; 5-LOX, 5-lipoxygenase; HXA_3, hepoxilin A_3; LTB_4, leukotriene B_4.

Table 2.

Condition	Adhered CFU expressed as: CFU/mL (x 10^4)					
Blank End1, apical Gc						
Gc apical reservoir	0	0	0	0	0	
End1 monolayer pre-treatment						
12-lipoxygenase activity inhibitor						
DMSO	6	1.7	23	4.4	3	2.1
CDC	3.6	1.7	19	10	4.5	9.1
5-lipoxygenase activity inhibitors						
DMSO	2.5	4.4	5.2	1.9		
Caffeic Acid	4.3	4.7	7.4	1.8		
DMSO	0.7	1.2	0.8	0.2	0.9	
Zileuton	1	1.9	1.3	0.2	0.6	
PKC inhibitor						
DMSO	18	5.3	7	6.5		
CCL	15	7.3	6	4		
PLA_2 inhibitors						
DMSO	6.6	7	3.6	7		
ONO-RS-082	5.9	4	0.7	6.8		
DMSO	9	13	25			
$cPLA_2$ inhibitor	6	9.2	22			
MeOH	6.7	2.4				
$sPLA_2$ inhib	5.5	1.4				
MRP2 inhibitor						
NT	10	5.7	16			
MK571	8	5.6	11			

COX-2 inhibitor							
DMSO	1	2	6				
NS-398	1	1.4	7.5				
Drug added to apical reservoir during PMN migration							
IL-8 blocking antibody							
DMSO	1.4	5.2	4.7				
anti IL-8 Ab	1.3	4.1	5.4				
Soluble epoxide hydrolase (sEH)							
NT	1.2	3.1	1.6	25			
sEH	1.2	2.8	1.9	18			
BLT_1 antagonist							
DMSO	1.9	1.6	25				
LY223982	2	5.2	23				
Gc strains							
Opa+, p+	8	2.9					
Opa-, p+	14	4.5	2.2	3	2.4	1.3	0.8
Opa+, p- (ΔPilE)	4.2	8.2					
Opa-, p- (ΔPilE)	6.1		2.9	1.9	1.9		0.5
Opa-, p- (ΔPilQ)						1.2	0.6
shRNA KD End1 monolayers							
Vector shRNA	2.1	3.8	1	6.3			
alox12b shRNA	3.3	3.6	1.3	11			
aloxe3 shRNA	2.4	4.3	1.3	4.8			
alox5 shRNA	2	3.6	1.2	3.5			

Table 2. Quantification of Gc CFU adherent to the apical aspect of End1 monolayers under different conditions. Abbreviations: CFU, colony forming unit; End1, End1 E6/E7 cells; Gc, *Neisseria gonorrhoeae*; CDC, Cinnamyl-3,4-dihydroxy-α-

cyanocinnamate; PKC, protein kinase C; CCL, Chletherine chloride; PLA_2, phospholipase A_2; MRP_2, multidrug resistance-associated protein 2; COX-2, cyclooxygenase-2; IL-8, interleukin-8; Ab, antibody; sEH, soluble epoxide hydrolase; BLT_1, leukotriene B_4 receptor 1; Opa, opacity-associated protein; p, type IV pilus; KD, knock-down.

Table 3.

Gene	**Protein**	Ct, run #1	Ct, run #2	ΔΔCt (Mock v. Inf. End1)
alox12b	12R-LOX	22.57796733	26.78505739	1.778593473, 3.468889695
aloxe3	eLOX-3	26.92871698	30.12622833	1.936754293, 2.597727382
alox12	12-LOX	33.57168134	34.33253161	0.920171706, 3.79546004
alox15	15-LOX	28.84508038	34.50465711	0.699709968, 2.981507276
alox5	5-LOX	31.9981877	33.63563601	1.012294, 1.764283713
gapdh	GAPDH	19.4422795	21.91153145	—

Table 3. Ct values for lipoxygenase expression evaluated in End1 cells by qRT-PCR. Abbreviations: Ct, cycle threshold; End1, Endocervical cell line End1 E6/E7; Inf., infected. Raw Ct values are shown from infected End1 monolayers.

Table 4.

Cell	Gene	Protein	Ct, run #1	Ct, run #2	ΔΔCt (Mock v. Inf. End1) or (Undiff. v. Diff. HL60)
Mock End1	*ltb4r*	BLT_1	31.08767955	31.4289697	—
Inf. End1	*ltb4r*	BLT_1	29.52616437	30.61082172	2.459867838, 1.94014313
Mock End1	*gapdh*	GAPDH	22.29762173	22.25770887	—
Inf. End1	*gapdh*	GAPDH	22.03468736	22.39572398	—
Undiff. HL60	*ltb4r*	BLT_1	28.88642502	29.63032182	—
Diff. HL60	*ltb4r*	BLT_1	29.24573358	29.2300574	9.282983043, 10.52789794
Undiff. HL60	*gapdh*	GAPDH	21.69534047	21.90018813	—
Diff. HL60	*gapdh*	GAPDH	24.69834232	24.89606921	—

Table 4. Ct values for BLT_1 expression evaluated in End1 or HL60 cells by qRT-PCR. Abbreviations: Ct, cycle threshold; End1, Endocervical cell line End1 E6/E7; Inf., infected; Undiff., undifferentiated; Diff., differentiated.

3. Discussion and Future Directions

3.1 Summary

Robust neutrophil influx has been described as a hallmark of gonococcal infection for centuries, with a description of gonorrhea appearing in the Bible and the writings of Hippocrates, for example (Edwards and Apicella, 2004). Despite characteristic neutrophilic inflammation to gonococcal infection, at the start of this dissertation work, the signals that drive neutrophil transepithelial migration at the level of the cervix were not understood. Multiple lines of evidence support that Gc manipulates the immune response to impede development of a protective adaptive immune response and instead skew the immune response towards a Th17 driven neutrophilic response (Jerse and Deal, 2013; Liu et al., 2012; Liu et al., 2014; Liu and Russell, 2011; Stevens and Criss, 2018). Importantly, our lab and others have shown that Gc can evade neutrophil killing mechanisms to survive in their midst (Ball and Criss, 2013; Criss et al., 2009; Criss and Seifert, 2008, 2012; Gunderson and Seifert, 2015; Handing and Criss, 2015; Johnson et al., 2015; Johnson and Criss, 2011, 2013; Juneau et al., 2015; Ragland et al., 2017; Reimer et al., 2016; Sarantis and Gray-Owen, 2012; Simons et al., 2005; Sintsova et al., 2014; Smirnov et al., 2014; Stohl et al., 2012; Stohl et al., 2005; Stohl et al., 2013). Gc also modulates neutrophil activation, for example contributing to increased release of ROS and granule components, and escaping from NETs (Johnson et al., 2015; Sarantis and Gray-Owen, 2007, 2012; Sintsova et al., 2014; Smirnov et al., 2014). We therefore posit that neutrophilic inflammation that is not sufficient to clear Gc can contribute to host cell damage. This is particularly relevant in the context of female infection, which is very frequently asymptomatic (Edwards and Apicella, 2004). Untreated and unresolved infection in women leads to continued inflammation and

severe clinical consequences including PID, tubal scarring, ectopic pregnancy, and in some cases dissemination and vertical transmission (Wiesner and Thompson, 1980).

My studies demonstrate a previously unappreciated role for epithelial- and neutrophil-derived eicosanoids, HXA_3 and LTB_4, in coordinating neutrophil transepithelial migration to Gc infection of physiologically relevant endocervical monolayers. While these eicosanoids have been shown to stimulate neutrophil influx to other important mucosal bacterial pathogens, Gc is unique in that it does not encode the T3SS or adherence factors used by other pathogens to stimulate HXA_3 and/or LTB_4 production (Boll et al., 2012a; Boll et al., 2012b; Criss et al., 2001; Kohler et al., 2002; Lee et al., 2000; Pazos et al., 2017; Silva et al., 2004; Zurawski et al., 2006). Additionally, the endocervix is distinct from other mucosal sites in that it marks a transitional zone between skin-like and mucosal epithelium. High 12-lipoxygenase activity had previously been reported in human cervix (Flatman et al., 1986). In the context of Gc infection of this unique mucosal site, my studies reveal a role for a different epithelial lipoxygenase, 12R-LOX in conjunction with eLOX-3, than has been implicated for other mucosal sites. In fact, excluding reports of involvement of the 12R-LOX pathway in psoriatic neutrophil-rich lesions (Boeglin et al., 1998), this is the first time that these specific 12-lipoxygenase enzymes have been implicated in inflammatory pathology, and the first time they have been implicated during a response to infection. Future work is needed to define how Gc stimulates eicosanoid pathways at this unique cite, and will potentially reveal therapeutic targets for inflammatory pathologies involving these enzymes. Additionally, since the eicosanoids implicated are involved in the context of a number of bacterial pathogens, we envision that some of the adjunctive therapeutic targets to mitigate neutrophilic inflammation revealed by this work will prove to be more broadly relevant.

This work has revealed several important outstanding questions surrounding Gc-stimulation of eicosanoid-driven transepithelial migration, which will be discussed below

(Figure 21). First, what are the Gc features that interact with endocervical cells to stimulate 12-lipoxygenase-dependent eicosanoid production? Second, does Gc interact with transmigrated neutrophils to further stimulate LTB_4 production? Third, are neutrophils differentially activated against Gc following neutrophil transepithelial migration? Fourth, how does neutrophilic inflammation in the context of Gc infection in the FRT contribute to epithelial cell damage? Finally, would targeting eicosanoid generation and/or signaling be beneficial in a relevant disease model of Gc infection and neutrophilic inflammation?

3.2 Three-component Gc-endocervical cell-neutrophil system to model neutrophil transepithelial migration to Gc

An important contribution of this work is the development of a model in which to study neutrophil transepithelial migration to Gc infection of a physiologically relevant endocervical cell monolayer. Before this work, the models used in the field to study neutrophil recruitment and interactions with Gc did not incorporate neutrophil transepithelial migration at the level of the cervix. Modeling Gc infection is complicated by the fact that Gc is a human-specific pathogen, requiring specific receptors on human cells in order to establish infection. The male urethral challenge model has been particularly useful in delineating the progression of infection and the immune response to Gc during male infection (Cohen and Cannon, 1999). However there are some important differences between male and female infection, including the receptors Gc uses to bind and enter host epithelial cells and the resulting pro-inflammatory responses (Edwards and Apicella, 2004). These differences preclude translation of findings in the male urethral challenge model to female infection and it is unethical to conduct experimental infections in women due to potential for serious clinical sequelae. Primary and

immortalized cell models used to study Gc interaction with host epithelial cells have illustrated many important bacterial factors for infection but do not model neutrophil influx. Additionally, immortalized cell lines, including cancer-derived cell lines, do not completely reflect the initial site of infection for Gc, the endocervix.

A mouse model of cervico-vaginal infection requires antibiotic and 17β-estradiol treatment to suppress the microbiota and neutrophil influx that would otherwise inhibit Gc colonization and sustained infection (Feinen et al., 2010; Jerse, 1999; Jerse et al., 2011; Song et al., 2008). However infection in women occurs throughout the menstrual cycle (Edwards and Apicella, 2004). Recently, development of a female mouse model of upper reproductive tract infection may provide a novel platform for studying neutrophilic inflammation and its consequences during Gc infection (Islam et al., 2016). In this model, Gc is delivered transcervically into the uterine horn and initial infection leads to PID (Islam et al., 2016). This model does not require antibiotics or estradiol and can therefore be used to study uterine pathology, including neutrophil influx and its consequences (Islam et al., 2016). Transcervical infection in this model leads to infection of both the uterine horns and corpus of the upper reproductive tract, as well as the cervix and vagina of the lower reproductive tract (Islam et al., 2016). Additionally, Gc is seen in association with luminal neutrophils (Islam et al., 2016).

For this work, we established a three-component Gc-endocervical cell-neutrophil system in order to specifically ask whether Gc-endocervical cell interactions are sufficient to stimulate neutrophil transepithelial migration. In this light, our three-component system is advantageous in that it provides a simplified platform in which to tease out a contribution of Gc-endocervical cell interactions in the absence of complicating contributions from resident immune and surrounding cells. However, there are several important caveats to the three-component system established in this work. First, End1 cells are an immortalized cell line, and while they maintain many important

tissue-specific phenotypes, they may not completely mimic endocervical cells, and lack for example the full context of cues from supporting cells, resident immune cells, and the microbiota of the adjacent lower FRT that they receive *in vivo*. Second, our three-component system does not incorporate resident immune and supporting cells, including dendritic cells and Th-17 cells, which contribute to the signals regulating neutrophilic inflammation to Gc during infection *in vivo*. Third, while our three-component system allows for investigation of neutrophil transepithelial migration across an endocervical monolayer, it does not incorporate signals from surrounding ectocervical and upper FRT epithelial cells, and it does not allow for the study of ascending Gc infection. In light of these caveats, future work will necessarily incorporate studies in mouse models of Gc infection and could also benefit from validation of our findings in primary endocervical cells as well as tissue explants.

Ongoing collaboration with the Gray-Owen lab will allow us to test the role of the lipid mediators implicated in our three-component system during Gc infection *in vivo*. Further, in collaboration with the Sexually Transmitted Disease Clinic at the Thomas Jefferson District Health Department, we will collect samples from Gc-infected patients to test for the presence of the signaling molecules implicated by our work in the context of infection. Importantly, despite important caveats, the three-component system developed in this work has provided a unique platform to interrogate cervical epithelial cell signaling pathways that are sufficient to stimulate neutrophil transepithelial migration to Gc. Therefore, future work will continually incorporate studies in this model while expanding to additional, more complex models of Gc infection and neutrophilic inflammation. To this end, future work in additional models will be done to validate our findings, to provide *in vivo* relevance for the pathways this work has implicated in neutrophil influx to Gc, and to test the therapeutic strategies we have proposed based on our findings.

3.3 Gc factors that stimulate endocervical production of a 12-lipoxygenase-dependent neutrophil chemoattractant

We observed that neutrophil transepithelial migration requires Gc to be in contact with endocervical cells (Figure 8). Two important findings from our studies support this observation. First, neutrophil transepithelial migration required Gc to be in contact with endocervical cells, so that Gc in the apical reservoir of an End1 monolayer that were not in contact with the epithelium did not stimulate migration (Figure 8). We confirmed that Gc was indeed not in contact with End1 monolayers via staining for Gc on End1 monolayers following neutrophil transepithelial migration and by CFU enumeration (Table 2). Second, Gc alone in the apical reservoir of an acellular transwell insert did not stimulate migration, indicating that Gc alone is not chemotactic for neutrophils (Figure 8). We conclude that neutrophil migration does not depend on a Gc-secreted component. This is significant as bacterial components, including fMLP, can stimulate neutrophil transmigration. However, our work suggests that Gc stimulates this process via a contact-dependent interaction with endocervical epithelial cells. There are some important caveats to these experiments. First, while our results suggest that a Gc-secreted component does not drive neutrophil migration, it is possible that Gc adherence to endocervical monolayers allows for the local delivery and/or increased local concentration of a secreted component close to the epithelial cell membrane. Second, given that epithelial cell monolayers provide a barrier to rapid diffusion of chemotactic gradients, a chemotactic gradient provided by Gc in the apical reservoir of an acellular insert may diffuse, limiting its ability to stimulate directed neutrophil migration. While these caveats are important to consider, our results support our leading hypothesis that a surface feature of Gc is important for stimulating neutrophil migration.

We found that neutrophil transepithelial migration is not dependent on two major Gc surface adhesins, Opa proteins and the type IV pilus (Figure 8). These results were surprising as both Opa proteins and the type IV pilus have been shown to interact with epithelial cells and to affect host cell signaling (Edwards and Apicella, 2004; Edwards and Butler, 2011; Merz and So, 2000). One caveat to our findings is that we only tested two pilus mutants, one lacking the major pilin subunit PilE and the other lacking the subunit comprising the pore through which the pilus is extruded, PilQ. While these mutants are non-piliated, minor pilin subunits and pilus-associated proteins including PilC, can still be expressed and a role for these components in stimulating neutrophil transepithelial migration has yet to be investigated. Another caveat is that there are a number of other known or putative adhesins on Gc that may contribute to stimulation of neutrophil transepithelial migration. Gc does not encode for the T3SS or adherence factors that other bacterial pathogens use to stimulate epithelial HXA_3 production (Criss et al., 2001; Lee et al., 2000; Pazos et al., 2017; Silva et al., 2004; Zurawski et al., 2006). Therefore, in order to understand how Gc stimulates endocervical 12-lipoxygenase-dependent neutrophil transmigration, future work will investigate the potential Gc surface feature(s) involved.

We cannot rule out an additional, non-exclusive hypothesis that gonococcal porin influences epithelial eicosanoid signaling. Porin translocates into host cell membranes (Wen et al., 2000). In support of this hypothesis, porin, in conjunction with the type IV pilus, has been shown to induce a calcium-flux in epithelial cells (Ayala et al., 2005). Interestingly, the calcium flux observed in response to porin is rapid, and subsequent effects of the type IV pilus are thought to be calcium- and porin-dependent as well as influenced by pilus retraction mediated by PilT (Ayala et al., 2005). We did not observe a decrease in neutrophil transepithelial migration to non-piliated Gc (lacking the major pilin subunit, PilE, or the pore subunit, PilQ) (Figure 19), suggesting that piliation is not

required. Purified porin can translocate to eukaryotic membranes to form channels and trigger calcium-flux (Gotschlich et al., 1987; Muller et al., 1999). Thus, one hypothesis is that a gonococcal porin induced-calcium flux contributes to PKC and/or $cPLA_2$ activation and subsequent release of AA from cellular membranes. However, porin is the most abundant protein in the gonococcal outer membrane and is essential for bacterial viability, making most attempted mutations lethal (Barlow et al., 1989; Gotschlich et al., 1987; van der Ley et al., 1991). Recently, some evidence has shown that certain portions of PorB may be mutable (Chen and Seifert, 2014), and alternatively, purified porin can be added to cells to stimulate certain signaling pathways (Chen and Seifert, 2014; Massari et al., 2000; Snapper et al., 1997; Stohl et al., 2013; Wen et al., 2000). Future experiments using immunofluorescence and/or membrane fractionation followed by western blotting could be used to look for the translocation of porin into End1 membranes following Gc infection and may prompt additional studies into a role for porin in stimulating epithelial eicosanoid signaling. An initial experiment would be to add purified porin to polarized End1 monolayers and assess for neutrophil transmigration.

TLR recognition of Gc factors is immunostimulatory. End1 cells express TLR1, -2, -3, -5, and -6 but do not express TLR4 or MD2 (Fichorova et al., 2002), the receptor complex that recognizes LPS. Therefore, it was not surprising that purified Gc LOS did not stimulate neutrophil migration (Appendix B, Figure 29A). While TLR4 is not thought to be expressed at the level of the endocervix in vivo (Fazeli et al., 2005), an important caveat to our system is that immortalized cell lines such as End1 cells may have altered expression of defining features and our model does not incorporate resident immune or surrounding cells that might express TLR4 and contribute to immune activation at the level of the cervix. Gc porin and the Lip lipoprotein have been shown to stimulate TLR2 (Fisette et al., 2003; Massari et al., 2002). Since End1 cells have been shown to express TLR2 (Fichorova et al., 2002), we conducted a pilot experiment where the TLR2 agonist

Pam2CSK4 (Pam2) or Pam3CSK4 (Pam3) was added to the apical surface of End1 monoalyers before assaying for neutrophil transepithelial migration, however we did not observe any migration to this stimulus alone (Reported in Appendix B, Figure 29B). Additionally, we could further investigate a role for a TLR2-dependent Gc-End1 interaction using NF-kB HEK-293 reporter cells expressing TLR2 in a 96-well plate format as previously described (Fisette et al., 2003; Massari et al., 2002). Since we observed production of IL-8 and IL-6 from Gc infected End1 monolayers (Figure 14), these studies would help define whether this response depends on TLR2 in this system. Notably, given that IL-8 was not important for stimulating neutrophil transepithelial migration in our three-component system, we postulate that production of these cytokines serves to recruit and activate neutrophils at the site of infection but not to drive transepithelial migration. A caveat to our interpretation is that our model does not incorporate neutrophil recruitment from the bloodstream through the interstitium; however IL-8 and IL-6 released in this system are still likely relevant for priming neutrophils (Borregaard, 2010; Condliffe et al., 1998; Fichorova et al., 2001; Nadeau et al., 2002; Ramsey et al., 1995; Waage et al., 1989).

Our studies ruled out a requirement for the type IV pilus and Opa proteins in stimulating neutrophil transepithelial migration. Instead, our results suggested that a trypsin-sensitive Gc surface factor(s) is required to stimulate robust neutrophil transepithelial migration (Figure 19). For this experiment, we used a protocol provided by the Sikora group to treat Gc with trypsin in order to identify surface exposed trypsin-sensitive surface proteins (Zielke et al., 2014). Importantly, trypsin-treated Gc adhered equally well to End1 monolayers and were equally viable compared to vehicle-treated Gc, as assessed by microscopy (Figure 19C). We sent trypsin- or vehicle-treated supernatants for mass spectrometric analysis, which revealed a hypothesis-generating list of potential targets enriched in trypsin-treated Gc supernatants (reported in Appendix

B, Table 5). A number of the targets identified had previously been identified by independent Gc proteomics approaches using trypsin treatment of bacteria to investigate trypsin-sensitive surface peptides (Zielke et al., 2014). Of the factors we have tested to date, we have not identified a single factor that is required to stimulate neutrophil transmigration. Our mass spectrometric analysis of trypsin-treated Gc supernatants provided a list of promising candidates (Table 5). Ongoing experiments will focus on continuing to examine Gc factors on this list. For some of the targets, we can reach out to colleagues in the field in order to acquire mutants. For other targets, we can generate mutants. Importantly, there may be multiple factors that contribute to stimulation of eicosanoid generation, thus future studies should examine this possibility by using strains deficient in multiple Gc factors with potentially additive effects.

An interesting facet of the work presented here is that since neutrophilic inflammation likely contributes to disease pathogenesis, and given that the signals driving neutrophil transepithelial migration to Gc were previously uncharacterized, our studies reveal potentially new insights into Gc pathogenesis. Of the *Neisseria* species, only *N. gonorrhoeae* and *N. meningitidis* are pathogenic, while there are many commensal *Neisseria*, including some that colonize the human nasopharynx (Criss and Seifert, 2012; Johnson, 1983; Liu et al., 2015). The pathogenic *Neisseria* classically elicit robust neutrophilic inflammation, however, commensal *Neisseria* can sometimes cause disease, including endocarditis and meningitis (Johnson, 1983). Therefore, an outstanding question in the field is whether stimulation of neutrophilic inflammation is restricted to pathogenic *Neisseria.* Importantly, the features that contribute to the disease-causing ability of the pathogenic *Neisseria* are likely multifactorial and have still not been fully characterized. An additional complementary approach to the work presented here would be to compare neutrophil migration to various pathogenic strains of *N. gonorrhoeae* to migration to commensal *Neisseria* strains. These experiments

could be paired with comparative genome-wide analyses of these species, as the genome sequences are publicly available, in order to create a candidate list of factors important in Gc-induced neutrophil transepithelial migration. It will be particularly informative to compare a candidate list of factors identified in Gc as opposed to commensal *Neisseria* to the list of trypsin-sensitive surface factors identified by mass spectrometric analysis. To this end, we have acquired commensal *Neisseria* strains available through the ATCC, and ongoing work on this project will investigate neutrophil transmigration to these strains compared to lab strains of pathogenic *N. gonorrhoeae*. This approach would help to identify proteins that are either present or absent in pathogenic compared to commensal *Neisseria.* One important caveat to this approach is that there might be variants/homologues of certain proteins in commensal *Neisseria* that differ from their counterparts in pathogenic *Neisseria* in ability to stimulate neutrophil transepithelial migration. To address this possibility, mutants of Gc could be generated that express the variant/homologue from nonpathogenic *Neisseria* and tested for ability to stimulate neutrophil migration. These studies would be particularly compelling as they have the potential to reveal novel Gc factors important in stimulating neutrophilic inflammation and disease pathogenesis.

One of the barriers to identifying the Gc factor(s) stimulating endocervical eicosanoid signaling pathways is lack of a high-throughput screening method. While neutrophil transmigration is a relevant biological read-out, a more high-throughput method will be required to evaluate the numerous potential Gc factors for a role in stimulating endocervical epithelial eicosanoid generation. Given that AA liberation is the rate-limiting step for HXA_3 generation and that we found that neutrophil migration across Gc-infected End1 monolayers required $cPLA_2$, it would be informative to screen by looking at PLA_2-activation. To this end, future studies could use a PLA_2 activity assay (Abcam) to measure PLA_2 activity in non-polarized End1 cells infected with Gc to identify

promising surface factors (Bhattacharjee et al., 2016). A caveat to this approach could be that non-polarized End1 cells might not respond to Gc interaction in the same way in which polarized End1 monolayers respond. In support of this method, however, we found that lipid extracted supernatants from infected, non-polarized End1 cells stimulated significantly more neutrophil migration across acellular inserts, indicating that the chemoattractant(s) produced by End1 cells in response to Gc can be produced by non-polarized cells. Follow-up studies would include western blotting to evaluate translocation of $cPLA_2$ to membrane fractions upon activation and subsequent neutrophil transmigration assays for promising targets. Identifying the Gc factor(s) that stimulate endocervical eicosanoid production is an essential next step to this work, as the results will provide important insights into disease pathogenesis.

3.4 Neutrophil activation influenced by transepithelial migration and Gc

Neutrophils circulating in the bloodstream are quiescent and become activated in the context of inflammatory signals, extravasation, and trafficking to the site of infection (Borregaard, 2010). In order to study Gc interaction with human neutrophils, we currently use IL-8 treated, adherent neutrophils to model neutrophil priming at the site of infection (Criss et al., 2009). In these studies, we treat neutrophils with 80 ng/mL recombinant human IL-8. Gc-infected polarized End1 monolayers produced ~0.225 ng/mL of IL-8 in the basolateral reservoir and ~0.085 ng/mL in the apical reservoir (Figure 14), which is less than we currently use to prime adherent neutrophils in our laboratory. However, neutrophils become increasingly activated as they transmigrate and may produce more IL-8 during this process. Neutrophils that have migrated through an epithelial cell monolayer can exhibit increased activity against pathogens, for example in the case of neutrophils that have migrated across an intestinal epithelial cell layer in response to

fMLP are more effective at killing *S.* Typhimurium (Nadeau et al., 2002). HXA_3 is a potent neutrophil chemoattractant, but unlike fMLP or LTB_4, it serves as a pure chemoattractant without activating neutrophils (Sutherland et al., 2000). Our data indicate that neutrophils produce LTB_4 following transmigration to apical Gc infection, which likely serves to further activate neutrophils in paracrine and autocrine fashion. Additionally, transmigrated neutrophils can adhere to the apical surface of the epithelium following transmigration, potentially interacting with bacteria (Brazil et al., 2010; Lawrence et al., 2003; Szabady and McCormick, 2013). In our studies, neutrophils were visualized on the apical membrane of Gc-infected End1 monolayers following transmigration by SEM and appeared protrusive, suggesting that neutrophils become activated during migration to Gc in this system. Preliminary imaging flow cytometry analysis also revealed Gc in association with neutrophils that had transmigrated to infection in our three-component system (reported in Appendix C, Figure 31). Before this work, there was not a model to examine Gc interactions with neutrophils that incorporated neutrophil transepithelial migration. Therefore our model provides an important system in which to study human neutrophil activation against Gc following transmigration.

It will be important to study neutrophil activation following transmigration to gonococcal infection for two reasons: (1) transmigrated neutrophils may be differentially activated against Gc and (2) transmigrated neutrophils may contribute to host epithelial cell damage via off-target effects of antimicrobial activity. In the next subsections, I will discuss outstanding questions, including whether neutrophil interaction with Gc following transmigration further stimulates neutrophil LTB_4 production (3.4.1), and whether neutrophils are differentially activated against Gc following transmigration (3.4.2). I will then discuss potential contributions of neutrophilic inflammation in response to Gc to epithelial cell damage in section 3.5. Additionally, we cannot rule out the possibility that

other components released by endocervical cells and/or neutrophils contribute to neutrophil activation in this system. For example, IL-6 is produced by mucosal epithelial cells during neutrophil transepithelial migration to *S.* Typhimurium and serves to further activate neutrophils (Nadeau et al., 2002). Our preliminary results indicate that IL-6 is released by Gc-infected End1 monolayers (Figure 14). Therefore, future experiments should test for additional components that might be present in this system, for example using Luminex multiplex assays to analyze supernatants from Gc-infected End1 monolayers with or without neutrophil migration.

3.4.1 *Potential Gc stimulation of eicosanoid production in neutrophils*

LTB_4 mediates long-range neutrophil chemotaxis (≥ 300 μm distances for up to 40 minutes, according to one report describing neutrophil swarming) (Afonso et al., 2012; Lammermann et al., 2013). Recently, it has been appreciated that this long-range chemotactic gradient is due to LTB_4 release from human neutrophils through exosomes, which contain both LTB_4 and the enzymes responsible for synthesizing LTB_4: 5-LOX, FLAP, and LTA_4 hydrolase (Majumdar et al., 2016) (Figure 7). LTB_4-containing exosomes then activate resting neutrophils by signaling through BLT_1 (Majumdar et al., 2016). This process is important for directional migration, providing a stable gradient of neutrophil-derived LTB_4 to amplify neutrophil migration (Majumdar et al., 2016). LTB_4 release via exosomes may be particularly physiologically relevant, as free LTB_4 would diffuse quickly and therefore form a relatively transient gradient compared to the stable gradient that would result from LTB_4 being carried in exosomes where it is protected from the aqueous environment.

Neutrophil exosome release requires neutrophil stimulation. Notably, stimulation of neutrophils with fMLP or ionomycin leads to increased release of exosomes containing LTB_4 and its synthetic enzymes (Andreu and Yanez-Mo, 2014; Majumdar et al., 2016). Recently, *P. aeruginosa* has been shown to use the bacterial protein, ExoU, which exhibits PLA_2 activity, to enhance neutrophil LTB_4 production (Pazos et al., 2017). However, whether ExoU results in increased exosome-mediated LTB_4 release specifically has not been examined. Given that we observed increasing amounts of LTB_4 following neutrophil migration to Gc (Figure 12), future studies should test the hypothesis that Gc stimulates neutrophil LTB_4 production and/or release of exosomes containing LTB_4 to amplify the neutrophil chemotactic gradient. In support of this hypothesis, we have shown that certain variants of Gc are activating for neutrophils. For example, Opa-expressing Gc interact with human neutrophils to stimulate phagolysosome formation (Johnson et al., 2015; Johnson and Criss, 2013), degranulation (A. Smirnov, unpublished observations), and ROS production (Criss and Seifert, 2008). Thus, future experiments should test whether Gc, and in particular Opa-expressing variants, initiate LTB_4-containing exosome release from neutrophils that is important for neutrophil transepithelial migration. Further, given that neutrophil $cPLA_2$ activity is required for LTB_4 production, an additional outstanding question is whether Gc stimulates neutrophil $cPLA_2$ activation and/or whether a Gc secreted factor mimics or enhances neutrophil $cPLA_2$ activation. To this end, future experiments should assay neutrophil $cPLA_2$ activation following interaction with Gc using a PLA_2 activity assay (Abcam) with follow-up analysis of $cPLA_2$ translocation to membrane fractions using a membrane fractionation protocol for neutrophils previously used in our lab (Smirnov et al., 2014). An important consideration is that Gc factors that influence neutrophil LTB_4 production may not be the same factors that stimulate epithelial HXA_3 production. Our lab is performing studies to measure LTB_4 production by adherent, IL-8 primed neutrophils compared to neutrophils

that have migrated through an epithelial cell layer, one hypothesis being that transepithelial migration provides additional priming steps required for this response. One important caveat to our three-component system is that it does not incorporate neutrophil migration from the vasculature through the interstitium and subepithelial space, which also serves to prime neutrophils. Importantly, understanding whether Gc further stimulates neutrophil LTB_4 production will provide additional insight into how this pathogen stimulates neutrophil influx at the site of infection.

3.4.2 *Neutrophil activation against Gc following transepithelial migration*

Our lab has developed a number of assays to examine the response of neutrophils to Gc. Preliminary results using imaging flow cytometry indicate that neutrophils associate with Gc following transepithelial migration in our three-component system (reported in Appendix C, Figure 31), and that neutrophils appear highly polarized and protrusive on both the apical surface of End1 monolayers (Figrue 9E) and on the bottom of tissue culture wells (apical chamber) following transmigration (by inverted light microscopy reported in Appendix C, Figure 30). Therefore, future studies will examine neutrophil activation and ability to kill Gc following transepithelial migration to Gc using imaging flow cytometry, fluorescence-based assays, degranulation and ROS production by flow cytometry, and neutrophil extracellular trap (NET) formation by confocal microscopy as detailed below.

In order to more thoroughly examine neutrophil phagocytosis and internalization of Gc following transepithelial migration, future experiments will use imaging flow cytometry to measure parameters including percent of neutrophils with associated (bound and internalized) Gc and percent of internalized Gc using the method developed in our lab (Smirnov et al., 2015). Imaging flow cytometry is extremely powerful, allowing

us to analyze tens of thousands of events per condition. These experiments can be followed by fluorescence-based assays used commonly in our lab (Criss et al., 2009) to measure Gc viability upon exposure to neutrophils that have transmigrated. While transmigration further activates neutrophils, given that viable Gc are recovered from neutrophil-rich patient exudates (Wiesner and Thompson, 1980), we hypothesize that some fraction of Gc will still survive in the presence of transmigrated neutrophils. Since the three-component system presented in this work incorporates neutrophil migration across a physiologically relevant endocervical monolayer, these future studies will allow us to more accurately model how Gc might evade neutrophil killing *in vivo*.

In addition to studies to examine Gc viability upon exposure to transmigrated neutrophils, the system developed in this work will also allow us to explore neutrophil activation in general following transepithelial migration to Gc. Notably, since robust neutrophil influx is not sufficient to clear Gc infection, and since we postulate that neutrophilic inflammation contributes to host epithelial cell damage and subsequent severe clinical sequelae in women, these experiments will examine neutrophil activity that has been associated with off-target damage to host cells. To this end, we will interrogate granule exocytosis, ROS production, and NET formation in transmigrated neutrophils.

Neutrophil antimicrobials are packaged in cytoplasmic granules. Upon stimulation, neutrophils mobilize their granules for exocytosis and delivery to phagolysosomes. Neutrophil granules include primary (azurophilic), secondary (specific), tertiary (gelatinase), and secretory granules, and each contain different components, including antimicrobials, PRRs, components of the NADPH oxidase, and protein markers (Borregaard et al., 2007; Pham, 2006). Granule mobilization to the plasma membrane follows a pattern by which secretory granules are released first, with increased activation required to mobilize tertiary, secondary, and finally primary granules (Borregaard et al.,

2007; Pham, 2006). Therefore, exposure of markers for primary and secondary granules on the neutrophil plasma membrane is a marker of activation. Granule exocytosis can be evaluated using flow cytometry for surface exposure of CD63, a primary granule marker, CD66b, a secondary granule marker, and loss of CD62L facilitated by matrix metalloproteases in tertiary granules (i.e. MMP9) using a protocol used by our lab (Ragland et al., 2017). These studies will allow us to compare granule exocytosis in neutrophils that have undergone transepithelial migration to Gc compared to suspension neutrophils (negative control, low-activation state), adherent IL-8 primed neutrophils, and neutrophils that have transmigrated to an imposed apical gradient of fMLP.

Gc efficiently evades ROS from IL-8 primed adherent neutrophils *in vitro* (Criss et al., 2009), indicating that non-oxidative neutrophil antimicrobial activities are primarily responsible for neutrophil activity against Gc. Notably, off-target ROS can contribute to significant off-target host cell damage. We can measure ROS on a population level using luminol-dependent chemiluminescence as commonly used in our laboratory (Criss and Seifert, 2008). However, given that in this system we can infect End1 monolayers with fluorescently-labeled (CFSE) Gc and then gate on neutrophils in association with Gc using flow cytometry, we can also measure ROS production on a single cell basis in neutrophils following transepithelial migration by assaying dihydrorhodamine-123 fluorescence by flow cytometry (Emmendorffer et al., 1990; Walrand et al., 2003). These studies will allow us to interrogate ROS production by neutrophils that have associated with Gc following transepithelial migration compared to ROS production by IL-8 primed adherent neutrophils that we typically study in our lab and may provide important insight into differences in ROS production in this more complete three-component model of neutrophil priming following transepithelial migration.

Finally, another neutrophil extracellular killing mechanism includes NETs. Importantly, NETs have been implicated in significant immunopathology in a number of

different pathological contexts, for example in lung pathology including that seen in cystic fibrosis (Kruger et al., 2015; Porto and Stein, 2016; Sabbione et al., 2017; Saffarzadeh et al., 2012; Thomas et al., 2012). Given that we are able to visualize neutrophils adhered to the apical surface of End1 monolayers following transmigration, we can also examine NET formation in transmigrated neutrophils by staining End1 monolayers for extracellular DNA and histones using confocal microscopy. Additionally, a recent study revealed that HXA_3 may play an additional role in promoting neutrophil-mediated damage in cystic fibrosis, where HXA_3 was shown to induce NETs from human neutrophils (Douda et al., 2015). This is interesting in light of our hypothesis that HXA_3 is produced apically by infected endocervical cells to stimulate neutrophil migration, suggesting there may also be a role for HXA_3 in promoting NETs and potentially epithelial cell damage mediated by NETs. All together, understanding the extent to which neutrophils are activated to release granule components, ROS, and NETs would provide important insight into the extent to which this activation might be able to contribute to off-target host cell damage.

3.5 Epithelial cell damage and potential adjunctive therapeutic targets

Neutrophil activity can potentially contribute to continued inflammation and cause significant off-target host cell damage. For example, as discussed in Chapter 1, neutrophil CAMPs, proteases, ROS, and DNA and histones contained in NETs serve as signals to enhance inflammation and can induce direct damage to host cells and/or extracellular matrix (Kruger et al., 2015; Mittal et al., 2014; Saffarzadeh et al., 2012; Verjans et al., 2016). NETs in particular have been implicated in a number of inflammatory pathologies including lung injury, atherosclerosis, rheumatoid arthritis, and systemic lupus erythematosis (SLE) (Kruger et al., 2015). Interestingly, HXA_3 was

recently shown to stimulate NET production (Douda et al., 2015). In addition to causing direct host cell damage, neutrophils also contribute to continued inflammation and neutrophil recruitment. For example, Gc has recently been shown to skew the immune response towards Th17-driven neutrophilic inflammation (Jerse and Deal, 2013; Liu et al., 2012; Liu et al., 2014; Liu and Russell, 2011). Th17 cells produce IL-17 and IL-8 to further recruit neutrophils. Neutrophils then produce CCL20 and CCL2 to recruit more Th17 cells, amplifying neutrophil influx (Pelletier et al., 2010). As discussed previously, neutrophil-derived LTB_4 also contributes to amplification of chemotactic gradients for neutrophils, and has been described as an effector promoting neutrophil swarming at sites of inflammation (Lammermann et al., 2013). At the site of inflammation, neutrophil derived products including MMPs (i.e. MMP9) cleave ECM into fragments that can stimulate continued immune cell recruitment (Kruger et al., 2015). Additionally, neutrophil-derived MMP-8 and -9 in combination with prolyl endopeptidase (PE) generate proline-glycine-proline (PGP), which mimics chemokines to recruit neutrophils (Weathington et al., 2006).

Interestingly, there are numerous mechanisms in place to maintain homeostasis, including the generation of both pro-inflammatory and pro-resolving lipid mediators from AA (Dennis and Norris, 2015; Dobrian et al., 2011) (Figure 5). Additional mechanisms also contribute to regulation of eicosanoid-driven neutrophil recruitment, including sEH, which inactivates the neutrophil chemotactic activity of HXA_3 (Morisseau and Hammock, 2013; Morisseau et al., 2012) (Figure 6B). Additionally, there is a recently appreciated dual role for LTA_4 hydrolase in both production of the potent neutrophil chemoattractant LTB_4 as well as in dampening neutrophil recruitment by inactivating PGP (Snelgrove et al., 2010). Therefore, the balance of pro-inflammatory and pro-resolving lipid mediators is important in responding to infection as well as in maintaining and returning to homeostasis.

Pro-inflammatory and pro-resolving bioactive lipids are likely to be important during infection and inflammation in general. However, a role for the interplay and/or disturbance of these processes in the context of Gc FRT infection has not yet been characterized. In addition to the previously discussed future studies to further characterize the signals generated in the context of Gc infection that recruit and activate neutrophils, future studies to investigate the regulation and/or dysregulation of pro-resolving factors in the context of Gc infection of the cervix would significantly expand our understanding of Gc pathogenesis. My work provides a model three-component system in which future studies can be conducted to investigate epithelial cell damage at the level of the cervix in response to neutrophil transepithelial migration. Further, recent development of a mouse model of upper FRT infection that results in neutrophil influx (Islam et al., 2016) will allow future work to validate our findings in the context of Gc infection *in vivo.* Importantly, our work has identified a number of promising adjunctive therapeutic targets for limiting deleterious neutrophilic inflammation in conjunction with antibiotics that could be tested *in vivo*.

3.5.1 *Neutrophil-induced epithelial cell damage*

Neutrophils are robustly recruited to the site of gonococcal infection, but are not sufficient to clear Gc (Wiesner and Thompson, 1980). Importantly, given that Gc can evade multiple arms of the neutrophil antimicrobial arsenal, neutrophil activities may then induce off-target host cell damage. While Gc components, including PG and LOS, as well as immune mediators, including TNFα have been shown to induce direct epithelial cell damage in models of upper FRT infection (Gregg et al., 1981; McGee et al., 1999; Melly et al., 1981; Melly et al., 1984), neutrophil-driven damage to FRT epithelial cells has never been directly investigated. Additionally very little is known

about whether Gc infection and subsequent neutrophil influx lead to epithelial cell damage at the level of the cervix. Gc has recently been shown to invade into subepithelial space by inducing exfoliation of columnar epithelial cells in the endocervix (Wang et al., 2017). Additionally, transmigration of a large number of neutrophils across epithelial monolayers in general leads to disruption of monolayer barrier function and can damage the epithelium (Ginzberg et al., 2001; Nusrat et al., 1997). The three-component system developed in this work will provide a valuable system in which to test for neutrophil-induced cervical epithelial cell damage. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) should be used to interrogate for overt signs of epithelial cell damage. In addition, we can use techniques discussed in Chapter 2 (Figure 9) to evaluate endocervical epithelial barrier function. These techniques to assess tight junctional integrity include confocal microscopy to evaluate markers of tight junctions, including ZO-1, measurement of paracellular flux of FITC-dextran, and monitoring changes in TEER. Neutrophil transepithelial migration, and specifically the concurrent disruption of tight junctions, leads to gaps in the epithelium that result in decreased junctional integrity, and subsequent reduction in TEER (Nash et al., 1987; Nusrat et al., 1997). During homeostasis and immune response, these changes are reversible and tightly regulated, with overall maintenance of epithelial barrier function (Nash et al., 1988). However, with robust, dysregulated neutrophil influx, epithelial damage can ensue (Nusrat et al., 1997). Robust neutrophil migration can lead to alterations in the levels of tight junctional proteins, for example down-regulation of ZO-1 in epithelial cells adjacent to migrating neutrophils (Kucharzik et al., 2001), in part mediated by neutrophil proteases including elastase (Ginzberg et al., 2001) and subsequent changes in tight junctional complex formation and integrity. The results of studies of barrier function and epithelial cell damage during neutrophil transepithelial

migration to Gc will be important for understanding gonococcal disease pathogenesis and associated pathology.

3.5.2 *Adjunctive therapeutic targets to limit neutrophilic inflammation in conjunction with antibiotic therapy*

A number of important mucosal pathogens promote neutrophilic inflammation by stimulating 12-lipoxygenase production of HXA_3. Our study highlighted involvement of 12R-LOX in conjunction with eLOX-3, the first time that these particular 12-lipoxygenase enzymes have been implicated in the context of infection. In fact, under normal physiological conditions, the function of 12R-LOX and eLOX-3 activity is to prevent transepithelial water loss via production of molecules derived from linoleic acid (LA), and mutations in the genes encoding these proteins are linked to dry skin disorders including autosomal recessive congenital ichthyosis (ARCI) and non-bullous congenital ichthyosiform erythroderma (NCIE) (Epp et al., 2007; Krieg et al., 2013; Mashima and Okuyama, 2015). In this epidermal barrier-supporting role, LA as opposed to AA is the preferred substrate for 12R-LOX (Mashima and Okuyama, 2015). Interestingly, some of the first reports of 12R-LOX in the context of disease highlight the accumulation of its product, 12R-HETE in psoriasis scales (Boeglin et al., 1998), suggesting that it may play a role during certain inflammatory states compared to in homeostasis. It is possible that a shift in the proportion of available LA to AA skews 12R-LOX and eLOX-3 activity towards oxidation of AA and production of HXA_3 in this inflammatory context. In fact, given that our studies revealed a role for endocervical $cPLA_2$, we posit that Gc interaction with endocervical cells stimulates PLA_2-mediated release of AA, which would shift this pool in favor of HXA_3 production. Interestingly, 12R-LOX has also been found to play a role in mucus production, influencing the expression of mucins, including

MUC5AC (Garcia-Verdugo et al., 2012). It remains to be investigated whether 12R-LOX affects cervical mucus via regulation of mucins, however this would be an interesting role for 12R-LOX in contributing to host defenses and homeostasis at this site.

A remaining experimental hurdle for this work will be to detect HXA_3 from Gc-infected End1 cells directly. HXA_3 is labile, and is quickly degraded in an acidic environment or by epoxide hydrolases (Morisseau et al., 2012; Pace-Asciak, 2015). While we could detect increased neutrophil transmigration across an acellular insert to lipid-extracted supernatants from infected compared to mock-infected non-polarized End1 cells, we have not yet been able to detect HXA_3 by mass spectrometry. One possibility that remains to be explored is whether or not HXA_3 could be esterified, and therefore attached to membranes in our system, as has been found for some eicosanoids (Maskrey et al., 2007; Morgan et al., 2009; Morgan et al., 2010a; Morgan et al., 2010b; Thomas et al., 2010). However, our findings that neutrophil transepithelial migration was sensitive to MRP2 inhibition would suggest that HXA_3 is indeed being released and extruded through MRP2 in a free/non-esterified form. An additional experimental hurdle is the difficulty of ionizing HXA_3 for detection by mass spectrometry. Future work to identify this lipid will therefore be done in close collaboration with our collaborator Christophe Morisseau (UC Davis) in order to try to detect HXA_3 by mass spectrometry. In addition, our collaborative work will also open avenues of investigation into the role of sEH in the context of Gc-endocervical cell infection. Epoxide hydrolases have not been characterized in the FRT. It would be interesting to characterize the epoxide hydrolase(s) present in our system, either soluble or membrane bound, as these studies might reveal important insight into EH-mediated regulation of HXA_3 in the context of infection at the level of the endocervix. Future studies should look at the effect of targeting the generation and/or stability of HXA_3, specifically by 12R-LOX and eLOX-3 on neutrophil influx to Gc-cervical infection.

The eicosanoid LTB_4 has been widely studied due to its role in common conditions including asthma (Behera et al., 1998; Jame et al., 2007; Mashima and Okuyama, 2015; Zaman et al., 2006). Particularly in asthma, while there appears to be a central role for cysteinyl leukotrienes in mediating bronchoconstriction, the role of LTB_4 is thought to be the recruitment of neutrophils and eosinophils that characterize severe asthma that is refractory to steroid treatment (Wenzel et al., 1997). In fact, the drug Zileuton, which targets the 5-LOX enzyme that produces LTB_4, has been used successfully in asthmatic patients (Israel et al., 1996; Israel et al., 1993; Nelson et al., 2007). 5-LOX is primarily expressed in leukocytes. While respiratory epithelial cells have been shown to express 5-LOX in the context of inflammation (Behera et al., 1998; Brock, 2005; Jame et al., 2007; Zaman et al., 2006), we did not find 5-LOX to be expressed in infected or uninfected End1 cells. Instead, our studies revealed a role for neutrophil 5-LOX in producing LTB_4 to amplify the chemotactic gradient for neutrophil transepithelial migration. However, epithelial LTA_4 hydrolases have been found produce LTB_4 via a transcellular process (Bigby et al., 1989; Fabre et al., 2002). We did not detect any LTB_4 production from infected End1 monolayers when there were no neutrophils added (Chapter 2), however it would be interesting if a portion of LTA_4 from neutrophils might be processed to LTB_4 via End1 LTA_4 hydrolase (Figure 7). To test a role for LTA_4 hydrolase in our system, future experiments should examine expression of LTA_4 hydrolase upon End1 monolayer infection and interrogate whether exogenous addition of LTA_4 (Cayman Chemical) leads to LTB_4 production from End1 monolayers. It may be necessary to test this question using both infected and uninfected End1 monolayers if LTA_4-hydrolase is only expressed in the context of infection. Additionally, future experiments could also use an LTA_4 hydrolase inhibitor and/or genetic knockdown of LTA_4 hydrolase in End1 cells to interrogate this question. This future work could

potentially reveal additional adjunctive therapeutic targets to reduce non-productive neutrophilic inflammation to Gc in combination with antibiotics.

The LTB_4-BLT_1 axis is important for amplifying local gradients of neutrophil chemoattractants to promote stable, long-range recruitment of neutrophils to sites of infection and inflammation (Afonso et al., 2012; Ford-Hutchinson et al., 1980; Lammermann et al., 2013). We postulate that targeting the BLT_1 receptor would be a particularly successful approach, potentially with fewer off-target effects than targeting eicosanoid generation, which could lead to severe side effects due to disturbances in lipid pathways. To this end, BLT_1 antagonists have already been tested in clinical trials (Liston et al., 1998), and have shown promising results in antagonizing pathology associated with LTB_4-BLT_1 signaling in mice (Li et al., 2015). Notably, there has already been significant clinical success in targeting leukotriene receptors, for example with leukotriene receptor antagonists including monteleukast and zafirlukast (Diamant et al., 1999; Kemp et al., 1998; Lazarus et al., 1997; Pearlman et al., 1999). Additionally, it has been noted that while BLT_1 is primarily expressed on myeloid cells, its expression can be inducibly upregulated in response to LPS, cytokines, and LTB_4 in certain cells (Qiu et al., 2006). Therefore, it would be interesting to investigate BLT_1 expression on endocervical cells in the context of Gc infection and neutrophilic inflammation, where we found significant production of LTB_4.

In light of the emergence of Gc strains that are resistant to multiple end-line antibiotic therapies and the considerable risk for off-target host cell damage from robust neutrophil influx and activation during gonococcal infection, there is an urgent need to develop new therapeutic strategies for this common sexually transmitted infection. My studies reveal a novel line of inquiry into adjunctive therapeutics that could be used in combination with antibiotics to mitigate the severe clinical complications associated with neutrophilic inflammation during female gonococcal infection. Adjunctive therapies that

dampen the immune response in combination with antibiotics are already used successfully in clinic for bacterial infections that stimulate robust deleterious inflammatory responses, for example the use of adjunctive dexamethasone in the treatment of bacterial meningitis (Barichello et al., 2015).

Future work in collaboration with the Gray-Owen lab, which has developed a mouse model of PID that elicits robust neutrophil recruitment (Islam et al., 2016), as well as the development of mice transgenic for CEACAM expression and exhibit robust neutrophil influx to lower FRT Gc infection (Sintsova et al., 2014), will allow us to translate our *in vitro* characterizations of these potential adjunctive therapeutic targets to a relevant *in vivo* model of Gc infection and neutrophilic inflammation. A major caveat to interfering with lipid signaling pathways is that perturbations in these pathways can lead to significant off-target effects (Figure 5). In fact, in preliminary experiments conducted in the Gray-Owen lab, we observed increased neutrophil influx to Gc following systemic inhibition of 5-lipoxygenase activity with Zileuton or 12-lipoxygenase activity with CDC. Given that perturbing lipid signaling pathways systemically carries substantial risk for off-target effects, we envision that targeting HXA_3 at the level of the cervix, either by inhibiting its generation or promoting its degradation via sEH, will be an important area of exploration. Perhaps even more attractive is the potential to target amplification of neutrophil influx by inhibiting LTB_4 production and/or signaling through the BLT_1 receptor.

Importantly, our work supports a strategy whereby these pathways could be targeted in the context of multiple important bacterial pathogens that stimulate a dysregulated neutrophil response that contributes to disease pathology. In particular, since the mucosal pathogens found to stimulate these pathways to date affect mucosal epithelial sites including the lung, intestine, and now the cervix, we envision a strategy by which therapeutics could be delivered directly to the site, via inhalers, timed-release

capsules, and/or vaginal suppositories, which would significantly limit off-target effects and/or side effects. In support of this strategy in the context of gonococcal FRT infection, the Russell group has recently reported that intravaginal administration of biodegradable microspheres carrying IL-12 during Gc infection promotes bacterial clearance, development of Gc-specific antibodies, and subsequent immune-mediated protection against re-infection for up to 6 months (Liu et al., 2018). Importantly, these microspheres remained in the mouse genital tract for multiple days, allowing slow-release of IL-12 over time (Liu et al., 2018). Human IL-12 is ~60 kDa (or 60,000 g/mol) and can be packaged into microspheres (Egilmez et al., 2003); recombinant sEH is ~64 kDa while BLT_1 antagonists, including LY223982 (502.6 g/mol) and CP-105,696 (428.5 g/mol) are closer to 0.5 kDa. Both sEH and BLT_1 antagonists could therefore potentially be packaged in microspheres for delivery.

3.6 Overall conclusions

Neutrophilic inflammation is the hallmark of gonococcal infection and likely contributes significantly to disease pathogenesis and clinical sequelae in women. However, before this work the signals coordinating neutrophil transepithelial migration to Gc at the level of the cervix were uncharacterized. My studies reveal that Gc-endocervical infection stimulates epithelial- and neutrophil-derived eicosanoid production to drive neutrophil transepithelial migration. This work uncovers a novel line of inquiry into Gc factors that influence epithelial and neutrophil eicosanoid production. My studies support a model in which Gc interacts with endocervical cells to stimulate HXA_3 production and apical secretion to drive initial neutrophil transepithelial migration. Neutrophil-derived LTB_4 signaling through BLT_1 on neutrophils then amplifies the chemotactic gradient, driving robust neutrophil transepithelial migration (Figure 21).

Importantly, the three-component Gc-endocervical cell-neutrophil system used in this work has revealed a role for endocervical- and neutrophil-derived eicosanoids during neutrophil influx to Gc. Future work in this model and in mouse models with our collaborators will allow us to further define the roles of these eicosanoids during Gc infection and resultant neutrophil influx. Importantly, this work and the future studies it has prompted will reveal previously unappreciated mechanisms of Gc disease pathogenesis and pathologies that drive severe clinical consequences, particularly in women. Continued investigation of the therapeutic targets revealed by my studies could lead to adjunctive therapies that would mitigate severe consequences of non-productive neutrophilic inflammation to Gc infection, particularly in women. Finally, continued interrogation of the Gc factor(s) that stimulate these eicosanoid pathways may reveal novel Gc virulence factors and inform potential vaccine and/or therapeutic antibacterial targets.

3.7 Figures for Chapter 3

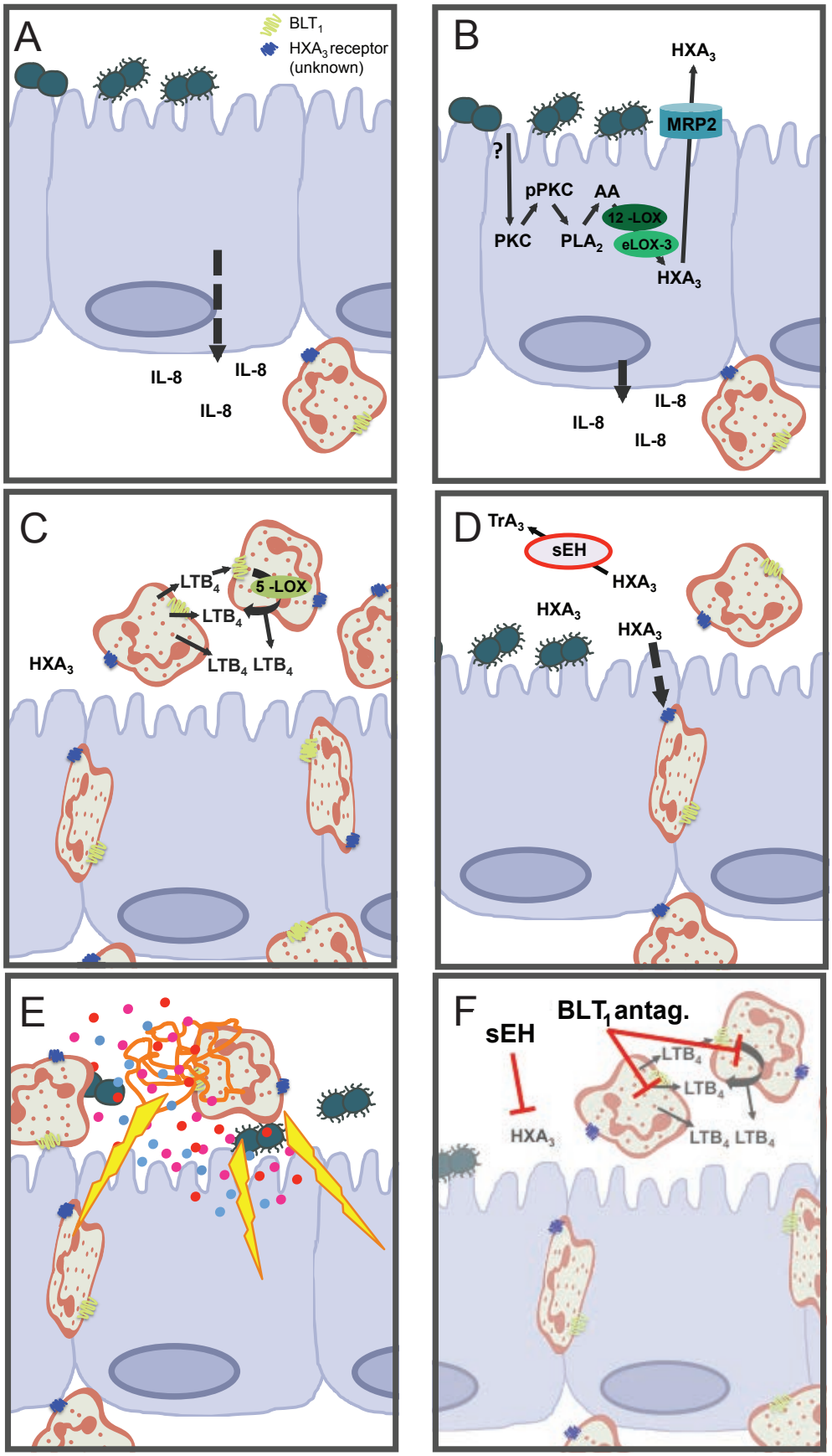

A
BLT1
HXA3 receptor (unknown)
IL-8
IL-8
IL-8
B
HXA3
MRP2
?
pPKC
AA
12 -LOX
PKC
PLA2
eLOX-3
HXA3
IL-8
IL-8
IL-8
C
LTB4
5 -LOX
LTB4
HXA3
LTB4 LTB4
D
TrA3
sEH
HXA3
HXA3
HXA3
E
F
sEH
BLT1 antag.
LTB4
LTB4
HXA3
LTB4 LTB4

Figure 21. Model of endocervical- and neutrophil-derived eicosanoids that stimulate neutrophil transepithelial migration to Gc. Gc interaction with endocervical cells stimulates IL-8 production that is primarily basolateral and likely serves to recruit and activate neutrophils at the site of infection (A). Gc-endocervical cell interactions stimulate endocervical cells to produce an apically directed 12-lipoxygenase-derived neutrophil chemoattractant, likely HXA_3 to stimulate initial neutrophil transepithelial migration (C) that required efflux through MRP2 (B) and was sensitive to sEH (D). This pathway was dependent on epithelial activation of PKC and $cPLA_2$ to produce the 12-lipoxygenase substrate AA (B). Transmigrating neutrophils then produced the 5-lipoxygenase product LTB_4, which formed an apical-to-basolateral gradient that amplified neutrophil recruitment (C). Robust neutrophil influx that is not sufficient to clear infection can lead to off-target host cell damage (E). This work reveals potential adjunctive therapeutic targets, whereby targeting HXA_3 and/or LTB_4 signaling at the level of the cervix could be a strategy to mitigate the deleterious effects of neutrophilic inflammation in conjunction with antibiotic therapy to resolve infection (F).

4. Appendix A: Investigation of neutrophil transmigration to Gc following four hours of infection

4.1 Introduction

There is a newfound appreciation for the role of epithelial cells in coordinating neutrophil transepithelial migration upon bacterial infection. In female patients, the initial site of gonococcal infection is the endocervix. As described in Chapter 2 of this dissertation, neutrophil transepithelial migration across Gc-infected polarized End1 monolayers is observed following only one hour of infection before addition of neutrophils and is coordinated by both epithelial and neutrophil derived eicosanoids. Previous model infection systems, however demonstrate that Gc interacts with epithelial cells via surface-exposed features including Opa proteins, type IV pili, and LOS to influence host cell signaling in additional ways, and that these interactions develop over extended time periods of infection (Edwards and Apicella, 2004; Edwards and Butler, 2011; Merz and So, 2000).

Following initial attachment mediated by type IV pili, and intimate attachment, mediated by Opa proteins, Gc forms microcolonies consisting of between 10-100 diplococci within 4-6 hours following infection (Merz and So, 2000). These microcolonies are motile structures that fuse and rearrange on epithelial cell surfaces (Higashi et al., 2007). Studies of Gc-epithelial cell interactions also revealed elongation of host cell microvilli, likely increasing surface area for bacterial interaction (Merz and So, 2000). Gc has also been shown to induce cortical plaques in infected epithelial cells with accumulation of epithelial signaling factors including PI-3 kinase, phosphotyrosine, actin, and ezrin, a phenomenon that requires the type IV pilus, and specifically retraction of the type IV pilus mediated by PilT (Higashi et al., 2007; Higashi et al., 2009; Lee et al., 2005;

Merz et al., 1999; Merz and So, 1997, 2000). Additionally, activation of NF-kB in infected epithelial cells has been shown to correlate with Gc microcolony dynamics and type IV pilus retraction (Dietrich et al., 2011; Muenzner et al., 2002; Naumann et al., 1997). Given that the majority of the field's understanding of Gc interations with host epithelial cells focused on later time points of infection, about 4-6 hours post infection, and given that Gc adherence to End1 cells was required to stimulate neutrophil transepithelial migration, we also investigated neutrophil migration following 4 hours of apical Gc infection.

4.2 Materials and Methods

4.2.1 *Bacterial strains and growth conditions*

Derivatives of Gc strain FA1090 (Ball and Criss, 2013) were used for these studies as follows: Opa+, p+ is an otherwise isogenic derivative of Gc strain FA1090 constitutively expressing OpaD (Ball and Criss, 2013); Opa-, p+ is an otherwise isogenic derivative of Gc strain FA1090 with in-frame deletions of the genes encoding the 11 Opa proteins (A-K) (Ball and Criss, 2013); Opa+, p- is an otherwise isogenic derivative of Gc strain FA1090 with an in-frame deletion of the *pilE* gene, and are non-piliated. Opa-, p- is an otherwise isogenic derivative of Gc strain FA1090 with in-frame deletions of the genes encoding the 11 Opa proteins (A-K) (Ball and Criss, 2013) and an in-frame deletion of the *pilE* gene, and are non-piliated. Gc was maintained on Gonococcal Medium Base (GCB) (BD Difco) with Kellogg's supplement I + II (Kellogg et al., 1963). For infection of epithelial cells, 16-18 hour overnight lawns were cultivated at 37^{o}C in 5% CO_2, swabbed into liquid medium, and resuspended in Hank's balanced salt solution

(HBSS; with Ca^{2+} and Mg^{2+}; Thermo Scientific) with 10 mM HEPES pH 7.4 and 5 mM $NaHCO_3$ ($HBSS^+$) at a concentration of 7 x 10^5 CFU/mL unless otherwise indicated.

4.2.2 *Human neutrophil isolation*

Venous blood was collected from healthy human donors who provided informed consent. All human subject research was conducted in accordance with a protocol approved by the University of Virginia Institutional Review Board for Health Sciences Research. Red blood cells were removed from heparinized blood by dextran sedimentation and neutrophils were isolated over a Ficoll-Hypaque gradient as previously described (Stohl et al., 2005) and resuspended in Dulbecco's phosphate buffered saline (DPBS; without Ca^{2+} and Mg^{2+}; Thermo Scientific) with 0.1% dextrose. Neutrophils were kept on ice and used within 1h following preparation. Neutrophil preparations were assessed by phase-contrast microscopy and contained >95% neutrophils. Replicate experiments used neutrophils from different donors on separate days.

4.2.3 *Cell culture*

Human End1/E6E7 (End1) cells (ATCC® CRL-2615™) were maintained and polarized End1 monolayers established on inverted Corning™ Transwell ™ inserts as described previously (Fichorova et al., 2005), with the following details. End1 cells were maintained in keratinocyte serum-free medium (KSFM, Life Technologies) supplemented with provided bovine pituitary abstract (BPE, 50 µg/mL) and recombinant epidermal growth factor (EGF, 0.1 ng/mL) as well as 0.4 mM $CaCl_2$ and 1X Antibiotic-Antimicotic

(ThermoFisher Scientific). End1 cells were maintained at 37^{o}C, 5% CO_2 in a humidified chamber and routinely tested negative for mycoplasma.

To establish polarized monolayers, End1 cells were seeded on inverted 6.5 mm diameter Corning^{TM} Transwell ^{TM} inserts with 3-µm pores (ThermoFisher Scientific) coated with 5 µg/cm^2 human type IV collagen (Sigma). 7 x 10^4 End1 cells were seeded on each coated inverted insert and allowed to attach for 5 hours before reverting into a 24-well tissue culture plate containing KSFM and incubated at 37^{o}C, 5% CO_2 for 8-11 days, after which time transepithelial electrical resistance (TEER) measured using an EVOM^2 voltometer (World Precision Instruments, Inc.) was ≥ 150 Ω cm^2 (Fichorova et al., 2005; Sathe and Reddy, 2014). Barrier function of End1 monolayers was also monitored by stable fluid resistance between apical and basolateral reservoirs and by measurement of 10 kDa FITC-dextran (Sigma) flux in the apical and basolateral compartments of End1 monolayers using a black F96 MicroWell^{TM} (ThermoFisher Scientific) and Wallac Victor-2 1420 Multilabel Counter 485/535nm (Perkin-Elmer).

4.2.4 *PMN Transmigration assays*

End1 monolayers were washed and inverted into a humidified chamber. Monolayers were apically infected with 7 x 10^5 Gc CFU equivalents (MOI=3) for 4 hr (unless otherwise indicated) at 37^{o}C, 5% CO_2. Neutrophil migration was assayed as previously described (Parkos et al., 1991), with the following details. Following infection, monolayers were washed extensively in HBSS$^+$ to remove non-adherent bacteria and reverted into 24-well tissue culture wells containing 1 mL HBSS$^+$ per well. 1 x 10^6 primary human neutrophils were added to the basal reservoir of Transwell^{TM} inserts and incubated for 2 hours at 37^{o}C, 5% CO_2. The number of neutrophils transmigrated was measured using a colorimetric assay for the neutrophil primary granule component

myeloperoxidase (MPO) using ABTS™ Chromophore Diammonium Salt (EMD Millipore), read on a Wallac Victor-2 1420 Multilabel Counter (Perkin-Elmer) using 1s readings at 405nm, and quantified relative to a standard curve for each donor and each experiment. For all PMN transmigration assays, migration was evaluated to buffer alone to evaluate monolayer integrity or an imposed apical gradient of fMLP (1 μM) to evaluate neutrophil migratory capacity, with all experiments included yielding > 1 x 10^5 PMNs transmigrated to fMLP. For each experiment, adhered CFU were enumerated by incubating infected monolayers with 1% Saponin, 5 mM $MgSO_4$ for 15 minutes at 37°C, 5% CO_2, disrupting membranes, and plating serial dilutions on GCB.

4.2.5 *Inhibitor treatment*

End1 monolayers were pre-treated with inhibitor, 50 μM Cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC) (Enzo Life Sciences), or vehicle (DMSO) control diluted in $HBSS^+$, incubated at 37°C, 5% CO_2 for 1hr, then thoroughly washed before infection. Inhibitors did not affect Gc adherence to End1 monolayers, as assayed by CFU enumeration, or End1 or PMN viability, as monitored by Trypan Blue (HyClone) exclusion. All conditions yielded >95% viable End1 cells. For all treatments, PMN migration towards an imposed apical gradient of fMLP was assayed to ensure neutrophils' intrinsic migratory ability was unaffected.

4.2.6 *Microscopy*

End1 monolayers on Transwell™ inserts were fixed in 4% paraformaldehyde (Electron Microscopy Sciences), permeabilized in 0.01% Triton-X 100 (ThermoFisher Scientific) and blocked in 10% normal goat serum in phosphate-buffered saline for 1 hr

at room temperature. Gc was labeled using a polyclonal rabbit anti-Gc antibody (Biosource) followed by an Alexa-Fluor 488-coupled goat anti-mouse immunoglobulin G (ThermoFisher Scientific). Phalloidin-555 and DAPI were used to stain actin and nuclei respectively. Confocal images were captured on a Zeiss LSM-700 confocal laser scanning microscope at the Advanced Microscopy Facility (AMF) at the University of Virginia. Z-stacks were captured and exported from Zen Black Edition (Zeiss) as TIF files.

4.3 Results

In order to investigate a role for Opa proteins and/or the type IV pilus in stimulating neutrophil transepithelial migration, we first examined adherence of these strains to End1 monolayers after 4 hours of infection as both Opa proteins and the type IV pilus have been shown to mediate adherence to epithelial cells. End1 monolayers were apically infected with Opa-expressing piliated (Opa+, p+), Opa-deficient piliated (Opa-, p+), Opa-expressing, non-piliated (Opa+, p-), or Opa-deficient non-piliated (Opa-, p-) derivatives of FA1090 Gc for 4 hours at a multiplicity of infection (MOI) of ~3 (~3 enumerated Gc CFU per End1 cell on an inverted Transwell). Loss of the type IV pili or Opa proteins reduced the percentage of the starting inoculum adhered to End1 monolayers following 4 hours of infection (Figure 22). Thus, we next investigated whether we could add increased amounts (MOI=6) non-piliated or Opa-deficient Gc to achieve equivalent adhered CFU. Doubling the inoculum of non-piliated or Opa-deficient Gc resulted in equivalent adhered Gc as visualized by confocal microscopy (Figure 23) and by CFU enumeration following infection (Table 2). We next assayed neutrophil transepithelial migration to equivalent adhered CFU of Opa-expressing piliated (Opa+, p+), Opa-deficient piliated (Opa-, p+), Opa-expressing, or non-piliated (Opa+, p-) Gc

(Figure 24). All three strains stimulated robust neutrophil transepithelial migration, however migration to Opa-deficient Gc was significantly reduced compared to Opa-expressing Gc (Figure 24). Further, pre-treatment of End1 monolayers with the 12-lipoxygenase activity inhibitor CDC also inhibited neutrophil transepithelial migration following 4 hours of Gc infection (Figure 25). From these results, we conclude that after 4 hours of Gc infection, neutrophil transepithelial migration does not require the type IV pilus or Opa proteins, and is sensitive to CDC inhibition of endocervical 12-lipoxygenase activity.

4.4 Discussion

Gc is known to interact with epithelial cells to promote the release of pro-inflammatory mediators to recruit and activate neutrophils (Criss and Seifert, 2012; Dietrich et al., 2011; Fichorova et al., 2001; Harvey et al., 2002; Muenzner et al., 2002; Naumann et al., 1998; Naumann et al., 1997). However, while pro-inflammatory cytokines recruit neutrophils through the extracellular matrix (McCormick et al., 1995), additional signals are required to drive them across epithelial layers. The 12-lipoxygenase-derived eicosanoid HXA_3 has been shown to drive this final neutrophil transepithelial migration to a number of important mucosal pathogens (McCormick et al., 1998; Mrsny et al., 2004; Szabady and McCormick, 2013). Inhibition of endocervical 12-lipoxygenase activity inhibited neutrophil transepithelial migration to a four hour infection with Gc, suggesting that even over longer periods of infection, 12-lipoxygenase activity is important for driving final neutrophil transepithelial migration. Interestingly, while Opa proteins were not required for neutrophil migration, Opa-expressing Gc did stimulate significantly more neutrophil migration compared to Opa-deficient Gc. Given that we found a requirement for Gc to be in contact with End1 cells in order to stimulate

neutrophil migration, it is possible that Opa proteins mediate intimate adherence of Gc to End1 cells over time, and thus increase association of Gc surface factor(s) that stimulate End1 eicosanoid production. Additionally, while we observed this effect of Opa expression after 4 hours of infection, there was not a significant difference in neutrophil migration to Opa-expressing compared to Opa-deficient Gc after just one hour of infection, as discussed in Chapter 2. Therefore, an effect of Opa and/or intimate interaction with endocervical cell membranes may only have an effect at later time points of infection. In conclusion, endocervical 12-lipoxygenase activity is still required to drive neutrophil transepithelial migration at later time points of infection. Further, Opa proteins may facilitate the Gc-End1 interactions, and future studies could investigate whether Opa proteins influence eicosanoid production through a role in adherence or through another unappreciated role.

4.5 Figures for Appendix A

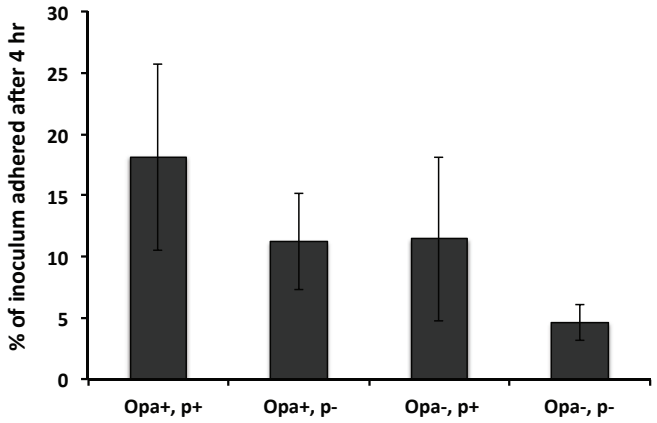


Figure 22. A higher percentage of Opa-expressing, piliated Gc adhere to polarized End1 monolayers compared to Opa-deficient or non-piliated Gc. Polarized End1 monolayers were infected with isogenic derivatives of strain of FA1090 Gc at an MOI=3. Adhered CFU were enumerated following 4 hr infection by incubating infected monolayers with 1% Saponin, 5 mM $MgSO_4$ for 15 minutes at 37°C, 5% CO_2, disrupting membranes, and plating serial dilutions on GCB. Results are plotted as the mean ± standard error of the mean for at least three independent experiments per condition. Abbreviations: Opa, opacity-associated protein; p, type IV pilus.

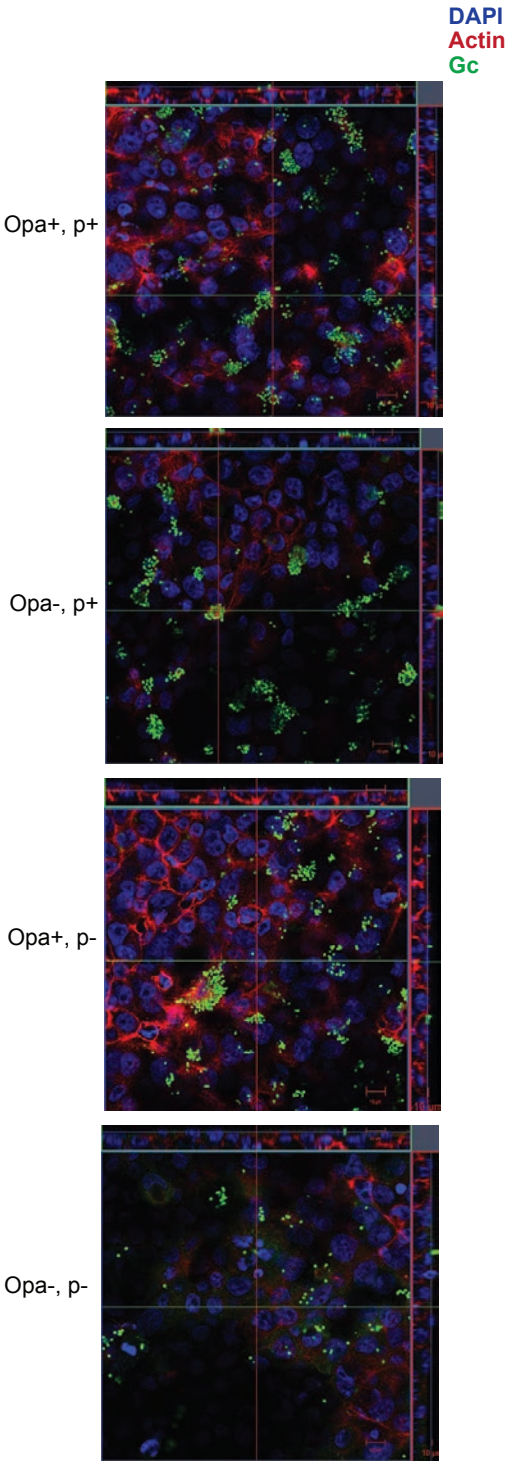

DAPI
Actin
Gc
Opa+, p+
Opa-, p+
Opa+, p-
Opa-, p-

Figure 23. Equivalent adhered Opa-deficient and non-piliated Gc following infection at dose-adjusted MOI. Polarized End1 monolayers were infected with isogenic derivatives of strain of FA1090 Gc at an MOI=3-6 (to normalize Gc-End1 association after 1hr). Representative images are shown of End1 monolayers with Gc (green), actin (red), and DAPI (blue). The confocal image was captured on a Zeiss LSM-700. Abbreviations: Gc, *Neisseria gonorrhoeae*; Opa, opacity-associated protein; p, type IV pilus.

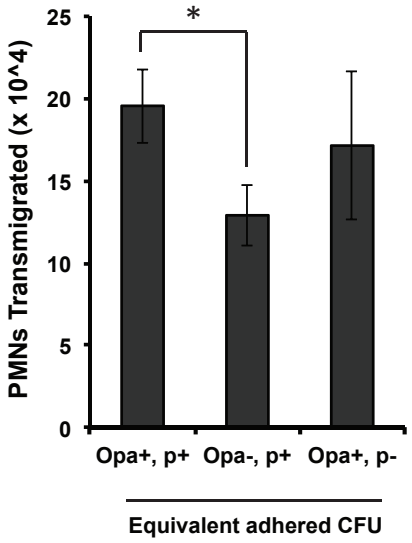


Figure 24. Equivalent adhered Opa-expressing Gc stimulate more robust neutrophil transepithelial migration compared to Opa-deficient Gc after 4 hours of infection. Polarized End1 monolayers were infected with isogenic derivatives of strain of FA1090 Gc at an MOI=3-6 (to normalize Gc-End1 association after 1hr). Neutrophil transmigration was assayed and results are expressed as a mean ± standard error of the mean for at least three independent experiments per condition. Statistics were calculated using a two-tailed, unpaired Student's *t*-test. **P* < 0.05. Abbreviations: Opa, opacity-associated protein; p, type IV pilus; PMN, polymorphonuclear cell/neutrophil.

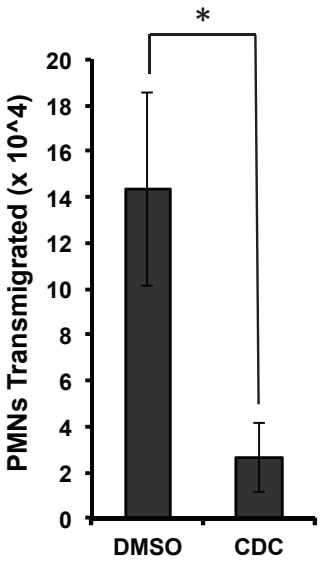


Figure 25. The 12-lipoxygenasae inhibitor CDC inhibits neutrophil transepithelial migration to Gc after 4 hours of infection. Polarized End1 monolayers were infected with a piliated, OpaD expressing of strain of FA1090 Gc at an MOI=3. Neutrophil transmigration was assayed and results are expressed as a mean ± standard error of the mean for at least three independent experiments per condition. Statistics were calculated using a two-tailed, unpaired Student's *t*-test. **P* < 0.05. Abbreviations: CDC, Cinnamyl-3,4-dihydroxy-α-cyanocinnamate; PMN, polymorphonuclear cell/neutrophil.

5. Appendix B: Investigating Gc surface factors for a role in stimulating neutrophil transmigration

5.1 Introduction

N. gonorrhoeae has been shown to interact with host epithelial cells to influence host cell signaling (Criss and Seifert, 2012; Dietrich et al., 2011; Fichorova et al., 2001; Harvey et al., 2002; Muenzner et al., 2002; Naumann et al., 1998; Naumann et al., 1997). As presented in Chapter 2 of this thesis, we had found that two of the prominent surface structures, Opa proteins and the type IV pilus, are not required to stimulate neutrophil transepitheilial migration to Gc. Additionally, stimulation of neutrophil transepithelial migration required Gc to be in contact with End1 monolayers and was influenced by a trypsin-sensitive surface factor(s). We therefore hypothesized that a trypsin-sensitive surface factor(s) contributes to stimulation of neutrophil transepithelial migration. Gc has an extensive surface proteome, which has recently begun to be analyzed using non-biased proteomics techniques including mass spectrometric analysis (Zielke et al., 2016; Zielke et al., 2014). In addition to revealing potential vaccine targets, we sought to examine these analyses in combination with our own analysis of trypsin-treated Gc supernatants in order to identify targets to test for involvement in stimulating neutrophil transepithelial migration. Additionally, there are a number of Gc factors that have previously been shown to interact with host cell signaling pathways, including a Gc-encoded PLD that is secreted upon Gc interaction with primary cervical cells and interacts with host epithelial Akt (Edwards et al., 2003), which we interrogated. The results of these studies have narrowed the list of Gc trypsin-sensitive surface factors that might contribute to stimulation of neutrophil transepithelial migration.

5.2 Materials and Methods

5.2.1 *Bacterial strains and growth conditions*

An otherwise isogenic piliated, OpaD-expressing (Opa+, p+) or piliated, Opa-deficient (Opa-, p+) derivative of Gc strain FA1090 with in-frame deletions of the genes encoding the 11 Opa proteins (A-K) (Ball and Criss, 2013) were used as parent strains for these studies. Gc was maintained on Gonococcal Medium Base (GCB) (BD Difco) with Kellogg's supplement I + II (Kellogg et al., 1963). For infection of epithelial cells, 16-18 hour overnight lawns were cultivated at 37^{o}C in 5% CO_2, swabbed into liquid medium, and resuspended in Hank's balanced salt solution (HBSS; with Ca^{2+} and Mg^{2+}; Thermo Scientific) with 10 mM HEPES pH 7.4 and 5 mM $NaHCO_3$ ($HBSS^{+}$) at a concentration of 7.6 x 10^{7} CFU/mL.

5.2.2 *Killing Gc before measuring neutrophil transepithelial migration*

Gc was either heat killed by incubation at 56^{o}C for 20 min, fixed for 15 min with 15% paraformaldehyde (PFA) (Electron Microscopy Sciences), or treated with 100% isopropanol (Fisher) for 5 minutes. Following killing, Gc were washed in PBS and then added to the apical surface of inverted polarized End1 monolayers.

5.2.3 *Mutant Gc strains*

Insertion deletions in Gc PLD (kan::pld) or Rmp (erm::rmp) were introduced into OpaD with bacterial gDNA from strains provided by Jennifer Edwards (PLD; Nationwide Children's Hospital) or Lee Wetzler and Ian Francis (Rmp) according to the previously

described spot transformation protocol (Stohl and Seifert, 2001). A *pilQ* null mutant was previously generated in our Opa-deficient background (Chen and Seifert, 2014; Drake and Koomey, 1995; Massari et al., 2000; Stohl et al., 2013) using gDNA, according to spot transformation protocol (Stohl and Seifert, 2001).

5.2.4 *Mass spectrometric analysis of trypsin- or vehicle-treated Gc supernatants*

OpaD Gc were swabbed from an 18 hr overnight lawn into GCBL. 5 x 10^8 CFU were treated with 40 ug/mL Sequencing grade Trypsin (Promega) or vehicle (Trypsin resuspension buffer, Promega) for 1hr at 37^{o}C on a rotating drum. Bacteria were then pelleted and the supernatant removed and processed. Supernatants were prepared for mass spectrometric analysis by passing over a Sulpeco Discovery DSC-18 SPE column (Sigma). To prepare the column, the column was first flushed with 50% acetonitrile, 1% acetic acid and then flushed with 1% acetic acid. The sample was loaded onto the column, washed with 1% acetic acid, and eluted with 80% acetonitrile, 1% acetic acid. Samples were transferred to the W.M. Keck Biomedical Mass Spectrometry Laboratory at the University of Virginia.

The following is copied from the report number 2135 from the W.M. Keck Biomedical Mass Specrtometry Laboratory: The sample was reduced with 10mM DTT in 0.1 M ammonium bicarbonate at room temperature for 0.5 h then alkylated with 50 mM iodoacetamide in 0.1 M ammonium bicarbonate at room temperature for 0.5 h. The sample was digested overnight at 37C with 1 µg trypsin in 50 mM ammonium bicarbonate. The sample was then acidified with acetic acid and dried to 15 µL for analysis.

The LC-MS system consisted of a Thermo Electron Velos Orbitrap ETD mass spectrometer system with an Easy Spray ion source connected to a Thermo 3 µm C18

Easy Spray column (through pre-column). 7 µL of the extract was injected and the peptides eluted from the column by an acetonitrile/0.1 M acetic acid gradient at a flow rate of 0.25 µL/min over 1.6 hours. The nanospray ion source was operated at 2.3 kV. The digest was analyzed using the rapid switching capability of the instrument acquiring a full scan mass spectrum to determine peptide molecular weights followed by product ion spectra (20) to determine amino acid sequence in sequential scans. This mode of analysis produces approximately 50000 MS/MS spectra of ions ranging in abundance over several orders of magnitude. Not all MS/MS spectra are derived from peptides.
The data were analyzed by database searching using the Sequest search algorithm against Uniprot *N gonorrhoeae* and Uniprot SwissProt.

5.2.5 *Details of LOS used from 1291 and TLR2 agonists*

Purified LOS from Gc strain 1291 was provided by Gary Jarvis (UCSF). LOS (5.7 ng), was added to the apical surface of inverted polarized End1 monolayers for 4 hours prior to assaying for neutrophil transepithelial migration. Pam2CSK4 (Pam2) or Pam3CSK4 (Pam3) (InvivoGen) was added to the apical surface of End1 monoalyers before assaying for neutrophil transepithelial migration.

5.2.6 *Human neutrophil isolation*

Venous blood was collected from healthy human donors who provided informed consent. All human subject research was conducted in accordance with a protocol approved by the University of Virginia Institutional Review Board for Health Sciences Research. Red blood cells were removed from heparinized blood by dextran sedimentation and neutrophils were isolated over a Ficoll-Hypaque gradient as

previously described (Stohl et al., 2005) and resuspended in Dulbecco's phosphate buffered saline (DPBS; without Ca^{2+} and Mg^{2+}; Thermo Scientific) with 0.1% dextrose. Neutrophils were kept on ice and used within 1h following preparation. Neutrophil preparations were assessed by phase-contrast microscopy and contained >95% neutrophils. Replicate experiments used neutrophils from different donors on separate days.

5.2.7 *Cell culture*

Human End1/E6E7 (End1) cells (ATCC® CRL-2615™) were maintained and polarized End1 monolayers established on inverted Corning™ Transwell ™ inserts as described previously (Fichorova et al., 2005), with the following details. End1 cells were maintained in keratinocyte serum-free medium (KSFM, Life Technologies) supplemented with provided bovine pituitary abstract (BPE, 50 µg/mL) and recombinant epidermal growth factor (EGF, 0.1 ng/mL) as well as 0.4 mM $CaCl_2$ and 1X Antibiotic-Antimicotic (ThermoFisher Scientific). End1 cells were maintained at 37°C, 5% CO_2 in a humidified chamber and routinely tested negative for mycoplasma.

To establish polarized monolayers, End1 cells were seeded on inverted 6.5 mm diameter Corning™ Transwell ™ inserts with 3-µm pores (ThermoFisher Scientific) coated with 5 µg/cm^2 human type IV collagen (Sigma). 7 x 10^4 End1 cells were seeded on each coated inverted insert and allowed to attach for 5 hours before reverting into a 24-well tissue culture plate containing KSFM and incubated at 37°C, 5% CO_2 for 8-11 days, after which time transepithelial electrical resistance (TEER) measured using an EVOM^2 voltometer (World Precision Instruments, Inc.) was ≥ 150 Ω cm^2 (Fichorova et al., 2005; Sathe and Reddy, 2014). Barrier function of End1 monolayers was also monitored by stable fluid resistance between apical and basolateral reservoirs and by

measurement of 10 kDa FITC-dextran (Sigma) flux in the apical and basolateral compartments of End1 monolayers using a black F96 MicroWell™ (ThermoFisher Scientific) and Wallac Victor-2 1420 Multilabel Counter 485/535nm (Perkin-Elmer).

5.2.8 *PMN Transmigration assays*

End1 monolayers were washed and inverted into a humidified chamber. Monolayers were apically infected with 2.3 x 10^6 Gc CFU equivalents (MOI=10) for 1 hr (unless otherwise indicated) at 37°C, 5% CO_2. Neutrophil migration was assayed as previously described (Parkos et al., 1991), with the following details. Following infection, monolayers were washed extensively in $HBSS^+$ to remove non-adherent bacteria and reverted into 24-well tissue culture wells containing 1 mL $HBSS^+$ per well. 1 x 10^6 primary human neutrophils were added to the basal reservoir of Transwell™ inserts and incubated for 2 hours at 37°C, 5% CO_2. The number of neutrophils transmigrated was measured using a colorimetric assay for the neutrophil primary granule component myeloperoxidase (MPO) using ABTS™ Chromophore Diammonium Salt (EMD Millipore), read on a Wallac Victor-2 1420 Multilabel Counter (Perkin-Elmer) using 1s readings at 405nm, and quantified relative to a standard curve for each donor and each experiment. For all PMN transmigration assays, migration was evaluated to buffer alone to evaluate monolayer integrity or an imposed apical gradient of fMLP (1 μM) to evaluate neutrophil migratory capacity, with all experiments included yielding > 1 x 10^5 PMNs transmigrated to fMLP. For each experiment, adhered CFU were enumerated by incubating infected monolayers with 1% Saponin, 5 mM $MgSO_4$ for 15 minutes at 37°C, 5% CO_2, disrupting membranes, and plating serial dilutions on GCB.

5.2.9 *Microscopy*

End1 monolayers on Transwell™ inserts were fixed in 4% paraformaldehyde (Electron Microscopy Sciences), permeabilized in 0.01% Triton-X 100 (ThermoFisher Scientific) and blocked in 10% normal goat serum in phosphate-buffered saline for 1 hr at room temperature. Gc was labeled using a polyclonal rabbit anti-Gc antibody (Biosource) followed by an Alexa-Fluor 488-coupled goat anti-mouse immunoglobulin G (ThermoFisher Scientific). Phalloidin-555 and DAPI were used to stain actin and nuclei respectively. Confocal images were captured on a Zeiss LSM-700 confocal laser scanning microscope at the Advanced Microscopy Facility (AMF) at the University of Virginia. Z-stacks were captured and exported from Zen Black Edition (Zeiss) as TIF files.

5.3 Results

We hypothesized that a Gc-endocervical cell interaction is important for stimulating neutrophil transepithelial migration. In support of this hypothesis, we showed that neutrophil transepithelial migration required Gc to be in contact with End1 monolayers (Figure 8).

5.3.1 *Paraformaldehyde-fixed Gc do not stimulate neutrophil transepithelial migration in contrast to heat-killed or isopropanol treated Gc*

An important question was whether or not the Gc-End1 interaction required live bacteria. In order to investigate this question, we examined neutrophil transepithelial migration following one hour of Gc at an equivalent MOI=10 that were killed by heat,

PFA fixation, or isopropanol treatment. In all cases, killed Gc adhered equally to the apical surface of End1 monolayers, as visualized by confocal microscopy (Figure 26). Interestingly, we found that while both heat-treated and isopropanol-treated Gc stimulated neutrophil transepithelial migration, PFA-treatment abolished neutrophil transepithelial migration (Figure 27). Based on these observations, we hypothesized that a surface feature, likely a protein, was being cross-linked by PFA and no longer able to interact with End1 cells.

5.3.2 *Two Gc surface factors identified by trypsin shaving, Rmp and PilQ, the secreted gonococcal PLD, and purified LOS are not required to stimulate neutrophil transepithelial migration*

Given that PFA-fixed Gc no longer stimulated neutrophil transepithelial migration, we next wondered whether a Gc surface protein might be important in stimulating neutrophil transepithelial migration. We found that Gc treated with trypsin prior to infection of End1 monolayers stimulate significantly less neutrophil transmigration (Figure 19). To identify the surface-exposed, trypsin-sensitive components, we sent supernatants from trypsin- or vehicle-treated Gc for mass spectrometric analysis. This analysis revealed 200 Gc proteins enriched in trypsin-treated Gc supernatants. From these data, we compiled a list of potential targets by cross-referencing our list with a previously published study of the surface proteome of our strain of Gc, FA1090 (Zielke et al., 2014) and with what is known from the literature (Table 5). From this prioritized list, we selected two components to test: PilQ and Rmp, which were enriched in trypsin-treated Gc supernatants (Table 5). PilQ is the secretin for the type IV pilus, and is essential for extrusion of the pilus (Drake and Koomey, 1995). The outer membrane protein reduction modifiable protein (Rmp) clusters with LOS and PorB in the outer

membrane of Gc and limits bactericidal antibody access to LOS, PorB, and potentially other Gc targets (Blake et al., 1989). These two targets were tested initially because we either had or could acquire strains with deletions in these two proteins from collaborators and generate these mutants in our FA1090 background. Mutants in either *pilQ* or *rmp* did not differ from parent Gc in their ability to stimulate neutrophil transepithelial migration (Figure 28). From these data, we can conclude that neither PilQ nor Rmp alone are required to stimulate neutrophil transepithelial migration. Continued work will test other prioritized targets (Table 5), including Ng-MIP, the pilus-associated protein and adhesin PilC, the putative pilus assembly protein PilN, Ng_1985, porin, and Ng_1981.

In addition to targets identified in trypsin-treated Gc supernatants, we also investigated some additional Gc factors known to affect epithelial cell signaling. We tested a role for a gonococcal-encoded phospholipase D (PLD) that has been shown to enter primary cervical epithelial cells and interact with host Akt leading to upregulation of CR3 on the surface of these cells (Edwards and Apicella, 2006; Edwards et al., 2003). We did not find a requirement for PLD in stimulating neutrophil transepithelial migration in our system (Figure 28). Further, purified LOS from strain 1291, provided by Gary Jarvis (UCSF), added to the apical surface of End1 monolayers did not stimulate neutrophil transepithelial migration (Figure 29A). From these results, we can conclude that Gc PLD is not required to stimulate neutrophil transepithelial migration across Gc-infected End1 monolayers and that up to 5.7 ng purified LOS does not stimulate neutrophil migration. Finally, addition of TLR2 agonists Pam2CSK4 (Pam2) or Pam3CSK4 (Pam3) End1 monolayers did not stimulate neutrophil transepithelial migration, indicating that this process may not depend on TLR2 (Figure 29B).

5.4 Discussion

Our mass spectrometric analysis of trypsin-treated Gc supernatants generated a large list of potential Gc factors to test. Many potential factors remain to be investigated on our prioritized list (Table 5), including PilC, a pilus assoiciated protein that has been implicated in host cell adhesion (Kirchner et al., 2005; Kirchner and Meyer, 2005; Rahman et al., 1997; Rudel et al., 1995) and is still expressed in mutants lacking *pilE*. Gonococcal MIP, a surface-exposed lipoprotein implicated in Gc survival from human neutrophils (Leuzzi et al., 2005) was also identified and remains to be tested in this system. This list of targets will inform future work in our lab to investigate the Gc surface factor(s) stimulating neutrophil transepithelial migration.

LOS has been found to be important in bacterial-bacterial and bacterial-epithelial Gc interactions, however neither End1 cells or primary endocervical cells express TLR4 (Fichorova et al., 2002), in agreement with other reports that TLR4 is not expressed at the level of the cervix (Fazeli et al., 2005). It is therefore not surprising that we did not see neutrophil transmigration in response to purified LOS. Importantly, this data supports previous observations in the FRT that TLR4 is not expressed at the level of the cervix, with expression beginning in the upper FRT, so as to titrate a robust immune response to pathogens that invade past the cervix (Fazeli et al., 2005; Fichorova et al., 2002).

Our results have allowed us to compile a list of targets, six of which have yet to be tested in our three-component system. Important caveats to this approach include that the Gc-endocervical cell interaction that stimulates epithelial eicosanoid production may be multifactorial and/or may involve additional bacterial factors apart from trypsin-sensitive surface features, as there was still some neutrophil migration to trypsin-treated Gc (Figure 19). Additionally, given that Gc has an extensive surface proteome, if none of the factors from our prioritized list, in isolation or in combination, account for Gc-

stimulated epithelial eicosanoid production, future work will require development of a more high-throughput method to screen potential Gc surface factors. Additionally, it is possible that stimulation of neutrophil transepithelial migration requires multiple Gc surface factors. Importantly, these studies have generated a list of factors to investigate and future work will inform our understanding of the role of the Gc surface factor(s) involved in gonorrheal disease pathogenesis.

5.5 Figures for Appendix B

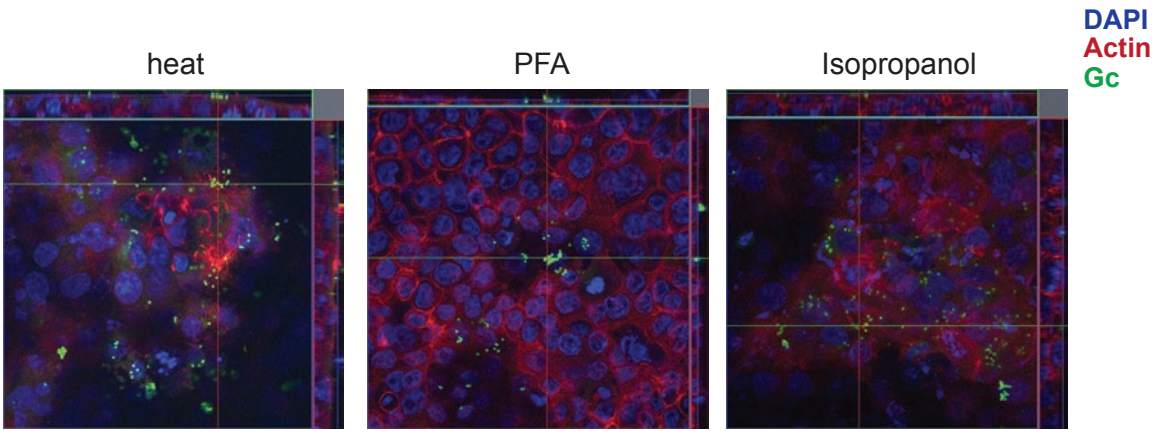


Figure 26. Gc killed by heat, PFA-fixation, or isopropanol treatment adhere to the apical surface of End1 monolayers. Gc was either heat killed, fixed with paraformaldehyde (PFA), or treated with isopropanol. Following killing, Gc were washed in PBS and then added to the apical surface of inverted polarized End1 monolayers for 1 hr at an equivalent to an MOI=10. Representative images are shown of End1 monolayers with Gc (green), actin (red), and DAPI (blue). The confocal image was captured on a Zeiss LSM-700. Abbreviations: Gc, *Neisseria gonorrhoeae*.

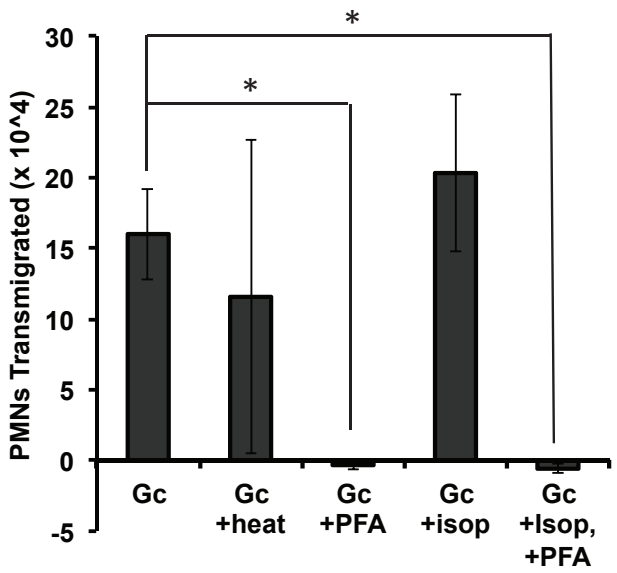


Figure 27. Compared to live Gc or Gc killed by heat or isopropanol treatment, PFA-fixed Gc do not stimulate neutrophil transepithelial migration. Gc was either heat killed, fixed with paraformaldehyde (PFA), or treated with isopropanol before being added to the apical surface of polarized End1 monolayers at an equivalent number to an MOI=10 for 1 hr. Neutrophil transmigration was assayed and results are expressed as a mean ± standard error of the mean for at least three independent experiments per condition. Statistics were calculated using a two-tailed, unpaired Student's *t*-test. *P < 0.05. Abbreviations: Gc, *Neisseria gonorrhoeae*; PMN, polymorphonuclear cell/neutrophil.

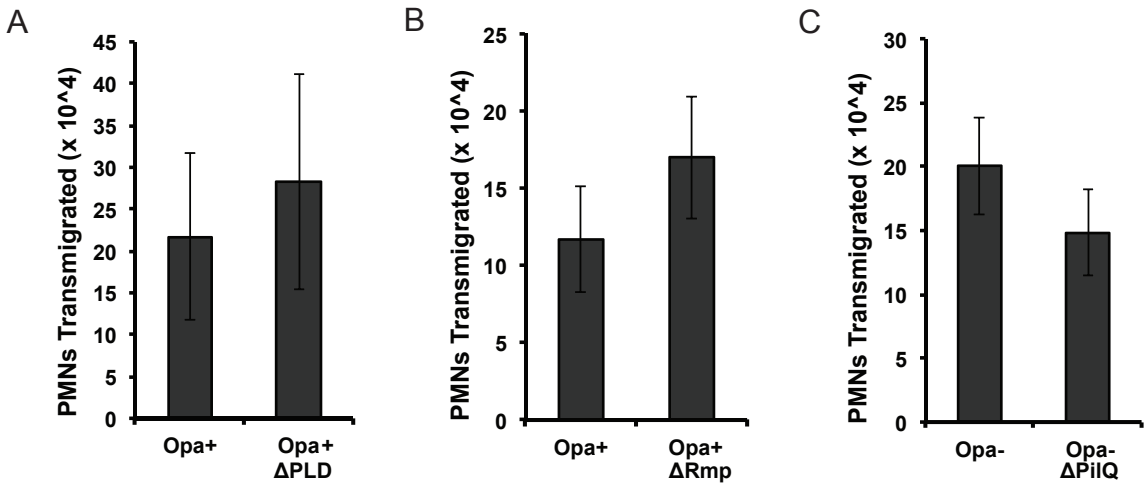


Figure 28. Gc lacking expression of PLD, Rmp, or PilQ still stimulate robust neutrophil transepithelial migration. Polarized End1 monolayers were infected with isogenic derivatives of strain of FA1090 Gc with mutations in PLD, Rmp, or PilQ at an MOI=10 for 1hr. Neutrophil transmigration was assayed and results are expressed as a mean ± standard error of the mean for at least three independent experiments per condition. Abbreviations: Gc, *Neisseria gonorrhoeae*; PMN, polymorphonuclear cell/neutrophil.

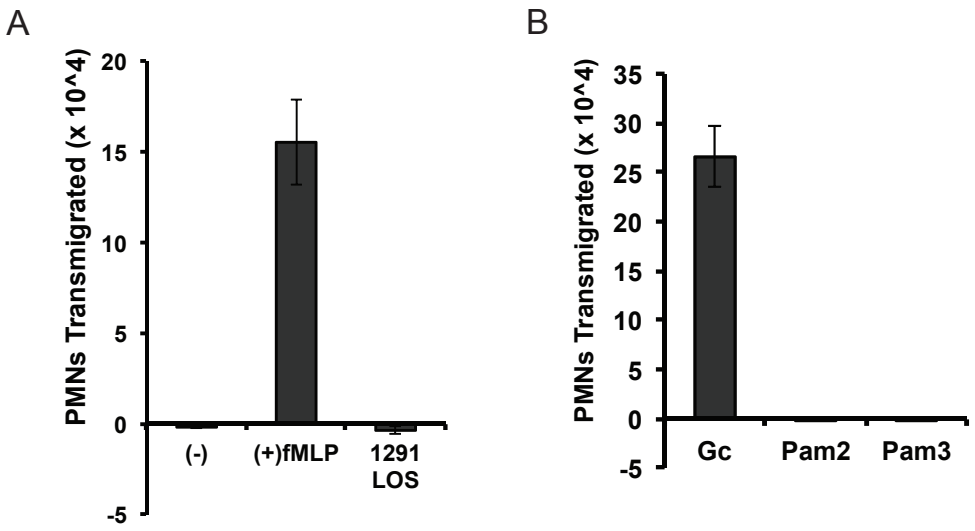


Figure 29. Purified Gc LOS and TLR2 agonists do not stimulate neutrophil transepithelial migration. Purified LOS from Gc strain 1291 (5.7 ng), was added to the apical surface of inverted polarized End1 monolayers for 4 hours prior to assaying for neutrophil transepithelial migration. Neutrophil transmigration was assayed and results are expressed as a mean ± standard error of the mean for at least three independent experiments per condition. Abbreviations: PMN, polymorphonuclear cell/neutrophil.

Table 5.

Identified protein	AltID (Gc ID)	MW	Tryp	Veh
Outer membrane protein PIII	rmp (NGO1577)	26 kDa	13	1
Peptidyl-prolyl cis-trans isomerase Ng-MIP	mip (NGO1225)	29 kDa	13	0
Pilus-associated protein	pilC (NGO0055)	110 kDa	38	0
Putative pilus assembly protein	pilN (NGO0097)	22 kDa	8	2
conserved hypothetical protein (possible hemolysin)	NGO1985	22kDa	5	0
pilQ	NGO0094	78 kDa	38	5
NG_1812	pIB (Neisseria-specific protein outer membrane protein porin IB)	37 kDa	11	1
NG_1981	lecA (adhesin protein)	20 kDa	1	0

Table 5. Prioritized targets identified from mass spectrometric analysis of trypsin-treated Gc supernatants.

6. Appendix C: Visualizing neutrophil transepithelial migration to Gc, neutrophil activation by lipid-extracted supernatants, and subsequent neutrophil activation against Gc

6.1 Introduction

An essential question highlighted during this work was whether we could visualize neutrophil activation during and/or after transepithelial migration and against Gc following transmigration. In order to assess these questions, we conducted some preliminary experiments using flow cytometry, imaging flow cytometry, and live time-lapse confocal microscopy.

6.2 Materials and Methods

6.2.1 *Bacterial strains and growth conditions*

Derivatives of Gc strain FA1090 (Ball and Criss, 2013) were used for these studies as follows: Opa+, p+ is an otherwise isogenic derivative of Gc strain FA1090 constitutively expressing OpaD (Ball and Criss, 2013); Opa-, p+ is an otherwise isogenic derivative of Gc strain FA1090 with in-frame deletions of the genes encoding the 11 Opa proteins (A-K) (Ball and Criss, 2013); Opa+, p- is an otherwise isogenic derivative of Gc strain FA1090 with an in-frame deletion of the *pilE* gene, and are non-piliated. Gc was maintained on Gonococcal Medium Base (GCB) (BD Difco) with Kellogg's supplement I + II (Kellogg et al., 1963). For infection of epithelial cells, 16-18 hour overnight lawns were cultivated at 37^{o}C in 5% CO_2, swabbed into liquid medium, and resuspended in

Hank's balanced salt solution (HBSS; with Ca^{2+} and Mg^{2+}; Thermo Scientific) with 10 mM HEPES pH 7.4 and 5 mM $NaHCO_3$ ($HBSS^+$) at a concentration of 7.6 x 10^7 CFU/mL.

6.2.2 *Human neutrophil isolation*

Venous blood was collected from healthy human donors who provided informed consent. All human subject research was conducted in accordance with a protocol approved by the University of Virginia Institutional Review Board for Health Sciences Research. Red blood cells were removed from heparinized blood by dextran sedimentation and neutrophils were isolated over a Ficoll-Hypaque gradient as previously described (Stohl et al., 2005) and resuspended in Dulbecco's phosphate buffered saline (DPBS; without Ca^{2+} and Mg^{2+}; Thermo Scientific) with 0.1% dextrose. Neutrophils were kept on ice and used within 1h following preparation. Neutrophil preparations were assessed by phase-contrast microscopy and contained >95% neutrophils. Replicate experiments used neutrophils from different donors on separate days.

6.2.3 *Cell culture*

Human End1/E6E7 (End1) cells (ATCC® CRL-2615™) were maintained and polarized End1 monolayers established on inverted Corning™ Transwell ™ inserts as described previously (Fichorova et al., 2005), with the following details. End1 cells were maintained in keratinocyte serum-free medium (KSFM, Life Technologies) supplemented with provided bovine pituitary abstract (BPE, 50 µg/mL) and recombinant epidermal growth factor (EGF, 0.1 ng/mL) as well as 0.4 mM $CaCl_2$ and 1X Antibiotic-Antimicotic

(ThermoFisher Scientific). End1 cells were maintained at 37^{o}C, 5% CO_2 in a humidified chamber and routinely tested negative for mycoplasma.

To establish polarized monolayers, End1 cells were seeded on inverted 6.5 mm diameter Corning^{TM} Transwell ^{TM} inserts with 3-µm pores (ThermoFisher Scientific) coated with 5 µg/cm^2 human type IV collagen (Sigma). 7 x 10^4 End1 cells were seeded on each coated inverted insert and allowed to attach for 5 hours before reverting into a 24-well tissue culture plate containing KSFM and incubated at 37^{o}C, 5% CO_2 for 8-11 days, after which time transepithelial electrical resistance (TEER) measured using an EVOM^2 voltometer (World Precision Instruments, Inc.) was ≥ 150 Ω cm^2 (Fichorova et al., 2005; Sathe and Reddy, 2014). Barrier function of End1 monolayers was also monitored by stable fluid resistance between apical and basolateral reservoirs and by measurement of 10 kDa FITC-dextran (Sigma) flux in the apical and basolateral compartments of End1 monolayers using a black F96 MicroWell^{TM} (ThermoFisher Scientific) and Wallac Victor-2 1420 Multilabel Counter 485/535nm (Perkin-Elmer).

6.2.4 *PMN Transmigration assays*

End1 monolayers were washed and inverted into a humidified chamber. Monolayers were apically infected with 2.3 x 10^6 Gc CFU equivalents (MOI=10) for 1 hr (unless otherwise indicated) at 37^{o}C, 5% CO_2. Neutrophil migration was assayed as previously described (Parkos et al., 1991), with the following details. Following infection, monolayers were washed extensively in HBSS$^+$ to remove non-adherent bacteria and reverted into 24-well tissue culture wells containing 1 mL HBSS$^+$ per well. 1 x 10^6 primary human neutrophils were added to the basal reservoir of Transwell^{TM} inserts and incubated for 2 hours at 37^{o}C, 5% CO_2. The number of neutrophils transmigrated was measured using a colorimetric assay for the neutrophil primary granule component

myeloperoxidase (MPO) using ABTS™ Chromophore Diammonium Salt (EMD Millipore), read on a Wallac Victor-2 1420 Multilabel Counter (Perkin-Elmer) using 1s readings at 405nm, and quantified relative to a standard curve for each donor and each experiment. For all PMN transmigration assays, migration was evaluated to buffer alone to evaluate monolayer integrity or an imposed apical gradient of fMLP (1 μM) to evaluate neutrophil migratory capacity, with all experiments included yielding > 1 x 10^5 PMNs transmigrated to fMLP. For each experiment, adhered CFU were enumerated by incubating infected monolayers with 1% Saponin, 5 mM $MgSO_4$ for 15 minutes at 37°C, 5% CO_2, disrupting membranes, and plating serial dilutions on GCB. For light microscopy images of transmigrated neutrophils, images were captured using a Nikon microscope affordable imaging camera attachment on an inverted light microscope.

6.2.5 *Time-lapse confocal microscopy*

For live, time-lapse confocal microscopy, purified human neutrophils were first loaded with the calcium-sensitive dye, fluo-4-acetoxymethyl ester (fluo-4-AM; Life Technologies) at a concentration of 1 μM at 37°C for 30 minutes, washed in $HBSS^-$, and resuspended in $HBSS^+$ with Tag-it Violet™ (BioLegend) at a concentration of 5 μM. End1 monolayers were stained with 20 μg/mL soy-bean lectin conjugated to Alexa Fluor® 647 (Invitrogen). Z-stack coordinates were set on the Zeiss LSM-700 at the AMF at UVA for each End1 monolayer. Neutrophils were then added to the basolateral reservoir of End1 monolayers and confocal images captured at 10X over the course of 2 hours. Individual Z projections were exported from Zen Black Edition (Zeiss) as TIF files.

6.2.6 *Imaging flow cytometric analysis of transmigrated neutrophils*

For infection of epithelial cells, 16-18 hour overnight lawns were cultivated at 37^{o}C in 5% CO_2, swabbed into liquid medium, and resuspended in Hank's balanced salt solution (HBSS; with Ca^{2+} and Mg^{2+}; Thermo Scientific) with 10 mM HEPES pH 7.4 and 5 mM $NaHCO_3$ ($HBSS^{+}$) at a concentration of 7.6 x 10^7 CFU/mL and labeled with 5 µg/mL carboxyfluorescein diacetate succinimidyl ester (CFSE) (Life Technologies) for 20 min at 37^{o}C. Following neutrophil transepithelial migration, neutrophils that had migrated to the bottom of the tissue culture well (apical chamber) were processed for imaging flow cytometry. Migrated neutrophils were lifted with 20 mM EDTA in PBS solution for 5 min om ice, collected into 1.5 ml tubes, and fixed with 2% PFA for 10 minutes on ice. Neutrophils were washed three times in PBS by pelleting (800 x g, 4 min, 12^{o}C) and resuspended in PBS and analyzed by imaging flow cytometry as previously described (Smirnov et al., 2015).

6.2.7 *Lipid extractions*

Lipids were isolated from End1 supernatants using solid phase extraction as previously described (Pazos et al., 2015; Tamang et al., 2012). Non-polarized End1 cells in 162-cm^2 flasks were infected with Gc at an MOI=100 in $HBSS^{+}$ or mock infected for 1 hr. Cells were then washed three times with $HBSS^{+}$ and incubated a further two hours in 15 mL $HBSS^{+}$. Infection supernatants were then collected, acidified to a pH 5, and extracted by solid-phase extraction using a Sulpeco Discovery DSC-18 SPE column (Sigma) and eluted with 3 mL methanol (Pazos et al., 2015; Tamang et al., 2012). Samples were then dried under nitrogen gas and stored at -80^{o}C until used. Prior to use, samples were re-suspended in $HBSS^{-}$.

6.2.8 *Calcium-flux by flow cytometry*

Purified human neutrophils were first loaded with the calcium-sensitive dye, fluo-4-acetoxymethyl ester (fluo-4-AM; Life Technologies) at a concentration of 1 μM at 37^{o}C for 30 minutes, washed in $HBSS^{-}$, and resuspended in $HBSS^{+}$. Calcium flux was then measured by flow cytometry as previously described (Pazos et al., 2015). Briefly, fluo-4-AM loaded human neutrophils were stimulated with ionomycin (1 μM; Thermo Fisher Scientific), LTB_4 (5 ng/mL; Enzo Life Sciences), $HBSS^{-}$, or lipid-extracted supernatants from End1 cells +/- Gc infection. Flow data was plotted as Fluo-4 intensity over time and analyzed with FlowJo software (Tree Star, Ashland, OR).

6.3 Results

Since we postulate that neutrophil activation and dysregulated response to Gc infection contributes to host cell damage and given that Gc can evade neutrophil killing in our current *in vitro* models and *in vivo*, we sought to determine whether we could use a number of different methods to visualize neutrophil transepithelial migration and neutrophil association with Gc following transmigration in our new three-component system. Further, since we hypothesize that the eicosanoid HXA_3 is produced by End1 cells in response to Gc, we examined and calcium flux in neutrophils following stimulus with lipid-extracted Gc-End1 infection supernatants. These preliminary studies will inform future studies looking at neutrophil activation during and following transepithelial migration.

6.3.1 *Neutrophils that have migrated through apically infected End1 monolayers spread out on the bottom of the 24-well plate tissue culture well*

Given that we can measure the number of neutrophils that have transmigrated through End1 cells to Gc infection using a colorometric assay for MPO, a primary granule component of neutrophils, we postulated that we would also be able to visualize transmigrated neutrophils in our system. Indeed, when we looked at the bottom of tissue culture wells (apical compartment), we observed neutrophils that had migrated to either fMLP or Gc, and appeared spread out and protrusive (Figure 30), suggesting that neutrophils become activated during migration in our system.

6.3.2 *Imaging flow cytometric analysis of neutrophils following migration through apically infected End1 monolayers reveal Gc in association with neutrophils*

Neutrophils that have transmigrated through an epithelial cell layer become increasingly activated against pathogens, for example exhibiting increased killing of *S.* Typhimurium following transepithelial migration to an imposed apical gradient of fMLP (Nadeau et al., 2002). Specifically, following neutrophil transmigration, neutrophil-released 5-AMP can be converted to adenosine on the epithelial cell surface and then stimulate epithelial production of IL-6 (Nadeau et al., 2002; Sitaraman et al., 2001). IL-6 can promote neutrophil degranulation (Nadeau et al., 2002). In addition, neutrophil activation during transepithelial migration is likely influenced by a combination of engagement of neutrophil cell surface proteins with ligands on epithelial cells (Liu et al., 2004) and epithelial and neutrophil production of inflammatory mediators such as IL-6 (Nadeau et al., 2002). Additionally, transmigrated neutrophils have been shown to adhere on the apical surface of epithelial monolayers before detaching, where they can

associate with bacteria (Brazil et al., 2010; Lawrence et al., 2003). We therefore hypothesized that we would observe neutrophils interacting with apically localized Gc following neutrophil transepithelial migration. We chose to use imaging flow cytometry for this purpose because we can analyze thousands of transmigrated neutrophils. Indeed, we were able to capture images of neutrophils that had associated Gc following neutrophil transepithelial migration (Figure 31A). We collected neutrophils following transepithelial migration to Opa+, p+, Opa-, p+, Opa+, p-, and Opa-, p- Gc and analyzed them using imaging flow cytometry (Figure 31). Of these strains, Opa+, p+ Gc exhibited the highest percentage of neutrophils associated with Gc, at 70%, with 57%, 39%, and 44.7% for the other strains respectively (Figure 31B), suggesting that Opa and/or pilus expression increases Gc association with transmigrated neutrophils. We also analyzed the number of CFSE+ spots per neutrophil using the Spot Count Wizard as previously described (Smirnov et al., 2015). Additionally, on average, neutrophils associated with Opa+, p+ contained 3 spots per cell, while the other strains contained 2.5 spots per cell. From this preliminary experiment, we can conclude that neutrophils can associate with Gc on the apical surface of End1 monolayers during transepithelial migration.

6.3.3 *Neutrophils can be visualized undergoing a calcium-flux as they transmigrate through End1 monolayers*

We sought to investigate whether we could visualize neutrophils transmigrating across End1 monolayers using time-lapse confocal microscopy during neutrophil transepithelial migration. Neutrophils pre-loaded with a calcium-sensitive dye were added to the basolateral reservoir of End1 monolayers immediately prior to imaging. We observed that neutrophils migrating across apically infected End1 monolayers in response to Gc infection or in response to an imposed apical gradient of fMLP (positive

control) underwent a calcium flux (Figure 32), implying specific signaling events that occurred as the neutrophils interacted with the activated epithelium and/or apically released chemoattractants.

6.3.4 *Measuring calcium-flux in neutrophils following a stimulus*

We had previously been able to show that lipid-extracted supernatants from infected End1 cells stimulate significantly more neutrophil transepithelial migration compared to lipid-extracted supernatants from uninfected End1 supernatants (Chapter 2). Based on the results in Chapter 2, our leading hypothesis is that End1 monolayers produce HXA_3 as the initial chemoattractant for neutrophils. HXA_3 has been reported to stimulate Ca^{2+} flux in neutrophils (Dho et al., 1990; Reynaud et al., 1995a; Reynaud et al., 1995b). Classical chemoattractants for neutrophils that also stimulate a Ca^{2+}-flux via pertussis-toxin sensitive activation of G-protein coupled receptors (GPCRs), directional chemotaxis, neutrophil degranulation, and ROS release include fMLP, chemokines (including IL-8), LTB_4, platelet activating factor (PAF), and complement fragments (C3 and C5a) (Liu et al., 2004). We observed neutrophil chemotactic activity in lipid-enriched supernatants from infected End1 cells, suggesting that a lipid mediator of neutrophil migration is involved (Figure 18). Further, we did not observe any LTB_4 production from End1 cells infected with Gc without neutrophils present, and IL-8 or IL-6 produced by infected End1 cells should be excluded from lipid-enriched supernatants. Thus we hypothesized that lipid-extracted supernatants from Gc-infected End1 cells, which are likely to contain HXA_3, initiate a calcium-flux in neutrophils, as reported for supernatants collected from epithelial cells infected with other pathogens shown to stimulate production of HXA_3 (Pazos et al., 2015). Stimulation of fluo-4-AM loaded neutrophils with ionomycin or LTB_4 induced a robust calcium flux, as indicated by the increase in Fluo-4

intensity immediately after addition of stimulus, and served as robust and relevant positive controls for detection of calcium-flux by flow cytometry (Figure 33). In two experiments, we were able to detect a calcium-flux in fluo-4-AM loaded neutrophils in response to lipid-extracted supernatants from infected compared to mock infected End1 cells (Figure 33). However, this result was not reproducible, meaning that for some experiments lipid-enriched supernatants did not stimulate a calcium flux. This was not due to experiment- or neutrophil donor- inability to produce a calcium flux, as ionomycin and LTB_4 stimulated robust calcium flux on those occasions. From these results, we conclude that HXA_3, which is also extremely labile, may be produced in small amounts and/or be difficult to liberate from the apical surface of Gc-infected End1 cells and that our preparations may need to be optimized for more robust recovery.

6.4 Discussion

An outstanding question from our previous work is whether neutrophils become differentially activated against Gc during neutrophil transepithelial migration. Neutrophil transmigration has been shown to increase neutrophil killing activity against another mucosal pathogen, *S.* Typhimurium (Nadeau et al., 2002). However, neutrophil activity has never been assayed following migration through Gc-infected endocervical monolayers. Importantly, since neutrophils are robustly recruited to Gc infection *in vivo*, and given that Gc can evade neutrophil killing, setting the stage for neutrophil-induced off-target host cell damage, investigating neutrophil activity following transmigration in general and against Gc remain important areas of investigation. Here we report that neutrophils that have transmigrated through Gc-infected End1 monolayers appear protrusive upon reaching the apical surface of End1 monolayers, associate with Gc from the apical surface of End1 monolayers, and undergo a calcium-flux during neutrophil

transmigration. These results suggest that neutrophils may become activated during neutrophil transepithelial migration to Gc. Further, the techniques used, including time-lapse confocal microscopy and imaging flow cytometry, will be useful in beginning to investigate some of the outstanding questions surrounding activation of neutrophils upon transmigration and their subsequent activity against Gc. Finally, the calcium-flux assay using flow cytometry will be useful for interrogating signals that induce a calcium-flux in neutrophils in this system. In conclusion, these studies provide important preliminary data to support the study of neutrophil activation in general and against Gc following transmigration across infected End1 monolayers.

6.5 Figures for Appendix C

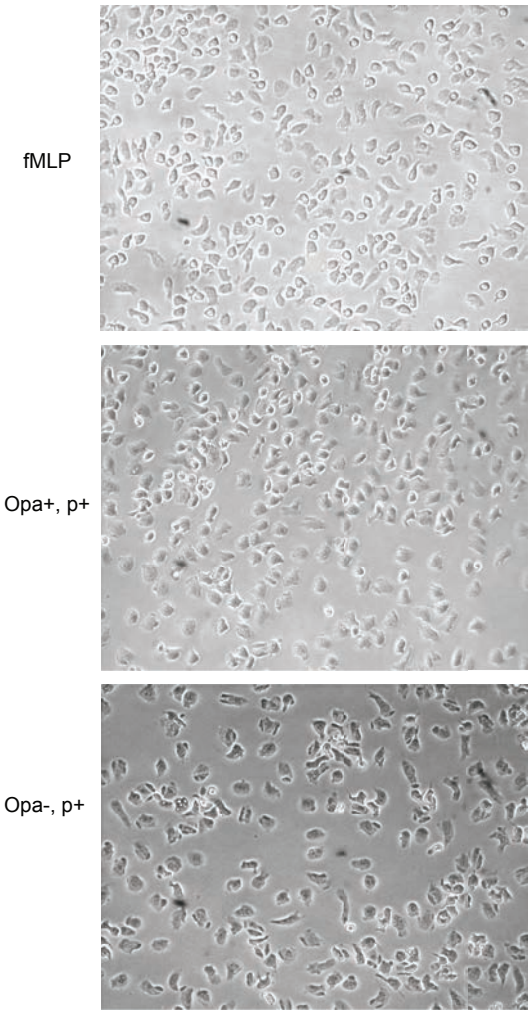
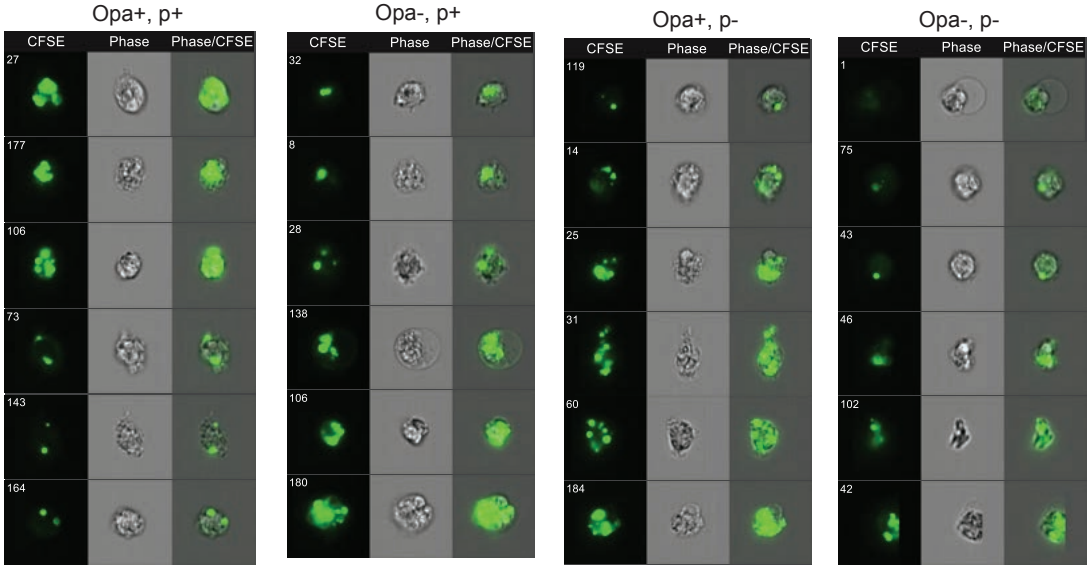


Figure 30. Light microscopy images of transmigrated neutrophils on the bottom of tissue culture wells. Polarized End1 monolayers were infected with isogenic strains of FA1090 Gc either expressing or deficient in Opa or type IV pili at an MOI=10. Neutrophils were allowed to migrate for 2 hr. Neutrophils that had transmigrated were visualized on the bottom of 24-well chamber (apical chamber) using a Nikon microscope affordable imaging camera attachment on an inverted light microscope. Abbreviations: Opa, opacity-associated protein; p, type IV pilus.

A



B

Opa+, p+

Spot Count_Spot(M02, Channel 2, Bright, 20.5, 2)

Population	Count	%Gated	Mean
Focused & Singlets	51	100	2.118
No bacteria & Focused & Singlets	15	29.4	0
CFSE+ & Focused & Singlets	36	70.6	3

Opa-, p+

Spot Count_Spot(M02, Channel 2, Bright, 20.5, 2)

Population	Count	%Gated	Mean
Focused & Singlets	49	100	1.449
No bacteria & Focused & Singlets	21	42.9	0
CFSE+ & Focused & Singlets	28	57.1	2.536

Opa+, p-

Spot Count_Spot(M02, Channel 2, Bright, 20.5, 2)

Population	Count	%Gated	Mean
Focused & Singlets	71	100	0.9718
No bacteria & Focused & Singlets	43	60.6	0
CFSE+ & Focused & Singlets	28	39.4	2.464

Opa-, p-

Spot Count_Spot(M02, Channel 2, Bright, 20.5, 2)

Population	Count	%Gated	Mean
Focused & Singlets	47	100	1.128
No bacteria & Focused & Singlets	26	55.3	0
CFSE+ & Focused & Singlets	21	44.7	2.524

Figure 31. Neutrophils can be visualized associated with Gc following transepithelial migration by imaging flow cytometry. Polarized End1 monolayers were infected with isogenic strains of FA1090 Gc either expressing or deficient in Opa or type IV pili at an MOI=10. Neutrophils were allowed to migrate for 2 hr. Representative images of neutrophils associated with Gc following transepithelial migration by imaging flow cytometry. Abbreviations: Opa, opacity-associated protein; p, type IV pilus.

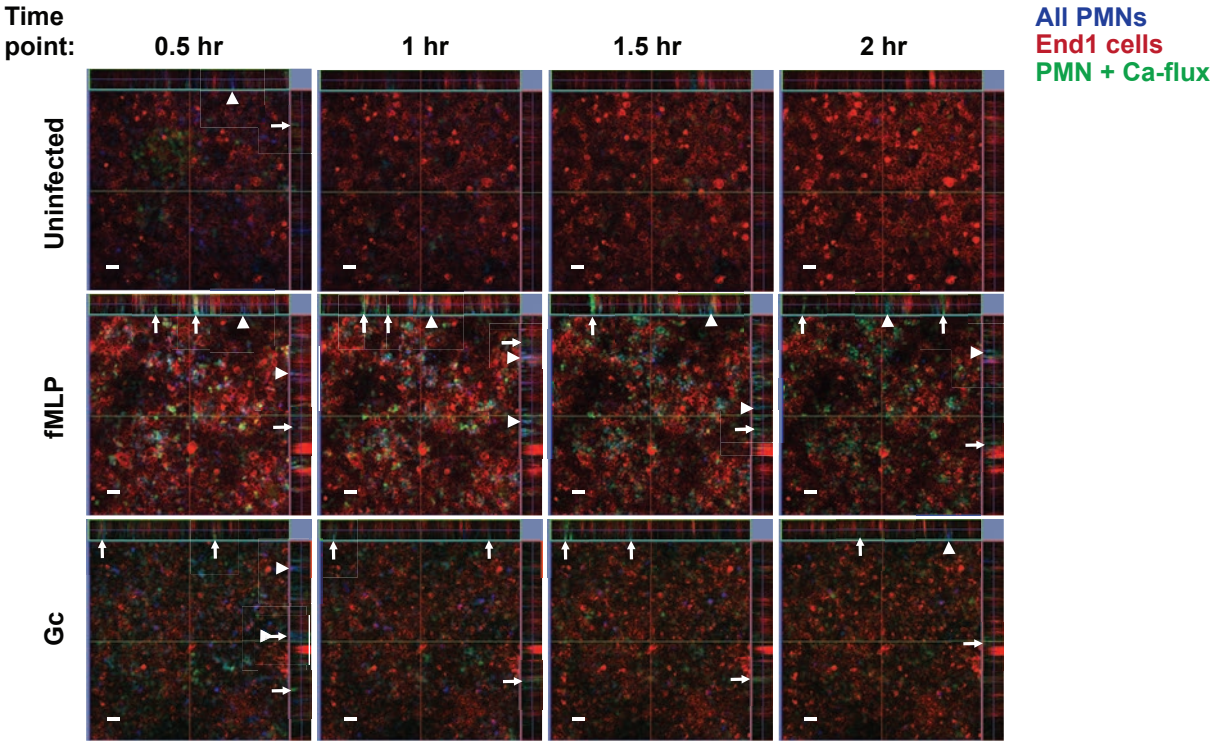


Figure 32. Time-lapse confocal microscopy reveals neutrophils undergoing a calcium-flux as they transmigrate to fMLP or Gc. Polarized End1 monolayers were infected with Gc at an MOI=10. Neutrophil transmigration was followed using live-cell multi-position time-lapse confocal imaging to capture z-stacks over time. Confocal images (10X) were captured on a Zeiss LSM-700. Scale bars represent 10μm. End1 cell borders were stained with lectin SBA (red), all neutrophils with Tag-it Violet (blue), and neutrophils undergoing a Ca-flux after stimulation/during transmigration were pre-loaded with the Ca-sensitive fluorescent dye Fluo-4AM (green). Arrows point to migrating neutrophils undergoing a Ca-flux (green) while arrowheads point to neutrophils. Representative images are from z position 5 (middle of the stack). Abbreviations: Gc, *Neisseria gonorrhoeae*; End1, End1 E6/E7 cells; PMN, polymorphonuclear cell/neutrophil.

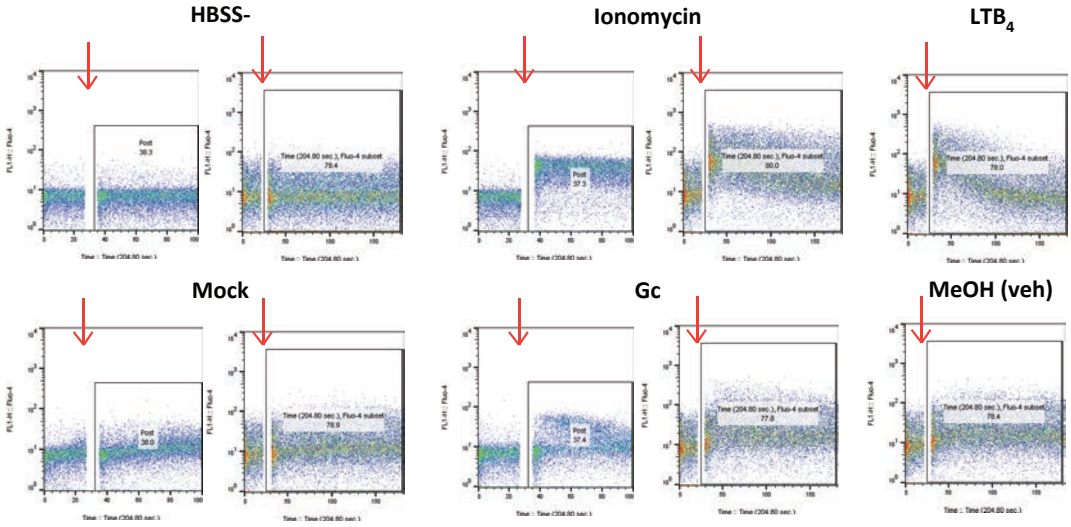


Figure 33. Calcium-flux in neutrophils by flow cytometry. Purified human neutrophils loaded with the calcium-sensitive dye fluo-4-AM were stimulated with ionomycin (1 μM), LTB_4 (5 ng/mL), $HBSS^-$, or lipid-extracted supernatants from End1 cells +/- Gc infection. Flow data was plotted as Fluo-4 intensity over time and analyzed with FlowJo software (Tree Star, Ashland, OR). Red arrows indicate time of stimulus. Abbreviations: Gc, *Neisseria gonorrhoeae*.

References

Afonso, P.V., Janka-Junttila, M., Lee, Y.J., McCann, C.P., Oliver, C.M., Aamer, K.A., Losert, W., Cicerone, M.T., and Parent, C.A. (2012). LTB4 is a signal-relay molecule during neutrophil chemotaxis. Dev Cell *22*, 1079-1091.

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

Andreu, Z., and Yanez-Mo, M. (2014). Tetraspanins in extracellular vesicle formation and function. Front Immunol *5*, 442.

Atack, J.M., Ibranovic, I., Ong, C.L., Djoko, K.Y., Chen, N.H., Vanden Hoven, R., Jennings, M.P., Edwards, J.L., and McEwan, A.G. (2014). A role for lactate dehydrogenases in the survival of Neisseria gonorrhoeae in human polymorphonuclear leukocytes and cervical epithelial cells. J Infect Dis *210*, 1311-1318.

Ayala, P., Wilbur, J.S., Wetzler, L.M., Tainer, J.A., Snyder, A., and So, M. (2005). The pilus and porin of Neisseria gonorrhoeae cooperatively induce Ca(2+) transients in infected epithelial cells. Cellular microbiology *7*, 1736-1748.

Ball, L.M., and Criss, A.K. (2013). Constitutively Opa-expressing and Opa-deficient neisseria gonorrhoeae strains differentially stimulate and survive exposure to human neutrophils. J Bacteriol *195*, 2982-2990.

Barichello, T., Collodel, A., Generoso, J.S., Simoes, L.R., Moreira, A.P., Ceretta, R.A., Petronilho, F., and Quevedo, J. (2015). Targets for adjunctive therapy in pneumococcal meningitis. J Neuroimmunol *278*, 262-270.

Barlow, A.K., Heckels, J.E., and Clarke, I.N. (1989). The class 1 outer membrane protein of Neisseria meningitidis: gene sequence and structural and immunological similarities to gonococcal porins. Mol Microbiol *3*, 131-139.

Beetham, J.K., Tian, T., and Hammock, B.D. (1993). cDNA cloning and expression of a soluble epoxide hydrolase from human liver. Arch Biochem Biophys *305*, 197-201.

Behera, A.K., Kumar, M., Matsuse, H., Lockey, R.F., and Mohapatra, S.S. (1998). Respiratory syncytial virus induces the expression of 5-lipoxygenase and endothelin-1 in bronchial epithelial cells. Biochem Biophys Res Commun *251*, 704-709.

Bhat, K.S., Gibbs, C.P., Barrera, O., Morrison, S.G., Jahnig, F., Stern, A., Kupsch, E.M., Meyer, T.F., and Swanson, J. (1991). The opacity proteins of Neisseria gonorrhoeae strain MS11 are encoded by a family of 11 complete genes. Mol Microbiol *5*, 1889-1901.

Bhattacharjee, A., Majumder, S., Das, S., Ghosh, S., Biswas, S., and Majumdar, S. (2016). Leishmania donovani-Induced Prostaglandin E2 Generation Is Critically Dependent on Host Toll-Like Receptor 2-Cytosolic Phospholipase A2 Signaling. Infection and immunity *84*, 2963-2973.

Bhowmick, R., Clark, S., Bonventre, J.V., Leong, J.M., and McCormick, B.A. (2017). Cytosolic Phospholipase A2alpha Promotes Pulmonary Inflammation and Systemic Disease during Streptococcus pneumoniae Infection. Infection and immunity *85*.

Bhowmick, R., Tin Maung, N.H., Hurley, B.P., Ghanem, E.B., Gronert, K., McCormick, B.A., and Leong, J.M. (2013). Systemic disease during Streptococcus pneumoniae acute lung infection requires 12-lipoxygenase-dependent inflammation. J Immunol *191*, 5115-5123.

Bigby, T.D. (1991). Transcellular metabolism of leukotrienes in the lung. Adv Exp Med Biol *314*, 235-250.

Bigby, T.D., Lee, D.M., Meslier, N., and Gruenert, D.C. (1989). Leukotriene A4 hydrolase activity of human airway epithelial cells. Biochem Biophys Res Commun *164*, 1-7.

Billker, O., Popp, A., Brinkmann, V., Wenig, G., Schneider, J., Caron, E., and Meyer, T.F. (2002). Distinct mechanisms of internalization of Neisseria gonorrhoeae by members of

the CEACAM receptor family involving Rac1- and Cdc42-dependent and -independent pathways. EMBO J *21*, 560-571.

Binnicker, M.J., Williams, R.D., and Apicella, M.A. (2003). Infection of human urethral epithelium with Neisseria gonorrhoeae elicits an upregulation of host anti-apoptotic factors and protects cells from staurosporine-induced apoptosis. Cellular microbiology *5*, 549-560.

Biosystems, A. (1997). Applied Biosystems Prism 7700 Sequence Detection System: user bulletin 2. Perkin-Elmer Corp, Norwalk, CT.

Blake, M.S., Blake, C.M., Apicella, M.A., and Mandrell, R.E. (1995). Gonococcal opacity: lectin-like interactions between Opa proteins and lipooligosaccharide. Infection and immunity *63*, 1434-1439.

Blake, M.S., Wetzler, L.M., Gotschlich, E.C., and Rice, P.A. (1989). Protein III: structure, function, and genetics. Clin Microbiol Rev *2 Suppl*, S60-63.

Blaskewicz, C.D., Pudney, J., and Anderson, D.J. (2011). Structure and function of intercellular junctions in human cervical and vaginal mucosal epithelia. Biol Reprod *85*, 97-104.

Boeglin, W.E., Kim, R.B., and Brash, A.R. (1998). A 12R-lipoxygenase in human skin: mechanistic evidence, molecular cloning, and expression. Proc Natl Acad Sci U S A *95*, 6744-6749.

Boll, E.J., and McCormick, B.A. (2012). A new understanding of enteroaggregative Escherichia coli as an inflammatory pathogen. Cell Adh Migr *6*, 413-418.

Boll, E.J., Struve, C., Sander, A., Demma, Z., Krogfelt, K.A., and McCormick, B.A. (2012a). Enteroaggregative Escherichia coli promotes transepithelial migration of neutrophils through a conserved 12-lipoxygenase pathway. Cellular microbiology *14*, 120-132.

Boll, E.J., Struve, C., Sander, A., Demma, Z., Nataro, J.P., McCormick, B.A., and Krogfelt, K.A. (2012b). The fimbriae of enteroaggregative Escherichia coli induce epithelial inflammation in vitro and in a human intestinal xenograft model. J Infect Dis *206*, 714-722.

Borregaard, N. (2010). Neutrophils, from marrow to microbes. Immunity *33*, 657-670.

Borregaard, N., Sorensen, O.E., and Theilgaard-Monch, K. (2007). Neutrophil granules: a library of innate immunity proteins. Trends Immunol *28*, 340-345.

Brazil, J.C., Lee, W.Y., Kolegraff, K.N., Nusrat, A., Parkos, C.A., and Louis, N.A. (2010). Neutrophil migration across intestinal epithelium: evidence for a role of CD44 in regulating detachment of migrating cells from the luminal surface. J Immunol *185*, 7026-7036.

Brock, T.G. (2005). Expression of 5-lipoxygenase in specialized epithelial cells of nasopharyngeal-associated lymphoid tissue. J Mol Histol *36*, 475-481.

Burch, C.L., Danaher, R.J., and Stein, D.C. (1997). Antigenic variation in Neisseria gonorrhoeae: production of multiple lipooligosaccharides. J Bacteriol *179*, 982-986.

Cahoon, L.A., and Seifert, H.S. (2011). Focusing homologous recombination: pilin antigenic variation in the pathogenic Neisseria. Mol Microbiol *81*, 1136-1143.

Cathcart, M.K. (2009). Signal-activated phospholipase regulation of leukocyte chemotaxis. J Lipid Res *50 Suppl*, S231-236.

Chan, J.M., and Dillard, J.P. (2016). Neisseria gonorrhoeae Crippled Its Peptidoglycan Fragment Permease To Facilitate Toxic Peptidoglycan Monomer Release. J Bacteriol *198*, 3029-3040.

Chan, J.M., and Dillard, J.P. (2017). Attention seeker: Production, modification, and release of inflammatory peptidoglycan fragments in Neisseria. J Bacteriol.

Chateau, A., and Seifert, H.S. (2016). Neisseria gonorrhoeae survives within and modulates apoptosis and inflammatory cytokine production of human macrophages. Cellular microbiology *18*, 546-560.

Chen, A., and Seifert, H.S. (2011). Neisseria gonorrhoeae-mediated inhibition of apoptotic signalling in polymorphonuclear leukocytes. Infection and immunity *79*, 4447-4458.

Chen, A., and Seifert, H.S. (2014). Saturating mutagenesis of an essential gene: a majority of the Neisseria gonorrhoeae major outer membrane porin (PorB) is mutable. J Bacteriol *196*, 540-547.

Chen, T., Grunert, F., Medina-Marino, A., and Gotschlich, E.C. (1997). Several carcinoembryonic antigens (CD66) serve as receptors for gonococcal opacity proteins. J Exp Med *185*, 1557-1564.

Chin, A.C., and Parkos, C.A. (2007). Pathobiology of neutrophil transepithelial migration: implications in mediating epithelial injury. Annu Rev Pathol *2*, 111-143.

Claesson, H.E., and Haeggstrom, J. (1988). Human endothelial cells stimulate leukotriene synthesis and convert granulocyte released leukotriene A4 into leukotrienes B4, C4, D4 and E4. Eur J Biochem *173*, 93-100.

Cohen, M.S., and Cannon, J.G. (1999). Human experimentation with Neisseria gonorrhoeae: progress and goals. J Infect Dis *179 Suppl 2*, S375-379.

Cole, J.G., Fulcher, N.B., and Jerse, A.E. (2010). Opacity proteins increase Neisseria gonorrhoeae fitness in the female genital tract due to a factor under ovarian control. Infection and immunity *78*, 1629-1641.

Condliffe, A.M., Kitchen, E., and Chilvers, E.R. (1998). Neutrophil priming: pathophysiological consequences and underlying mechanisms. Clin Sci (Lond) *94*, 461-471.

Connell, T.D., Shaffer, D., and Cannon, J.G. (1990). Characterization of the repertoire of hypervariable regions in the Protein II (opa) gene family of Neisseria gonorrhoeae. Mol Microbiol *4*, 439-449.

Criss, A.K., Katz, B.Z., and Seifert, H.S. (2009). Resistance of Neisseria gonorrhoeae to non-oxidative killing by adherent human polymorphonuclear leucocytes. Cellular microbiology *11*, 1074-1087.

Criss, A.K., and Seifert, H.S. (2006). Gonococci exit apically and basally from polarized epithelial cells and exhibit dynamic changes in type IV pili. Cellular microbiology *8*, 1430-1443.

Criss, A.K., and Seifert, H.S. (2008). Neisseria gonorrhoeae suppresses the oxidative burst of human polymorphonuclear leukocytes. Cellular microbiology *10*, 2257-2270.

Criss, A.K., and Seifert, H.S. (2012). A bacterial siren song: intimate interactions between Neisseria and neutrophils. Nat Rev Microbiol *10*, 178-190.

Criss, A.K., Silva, M., Casanova, J.E., and McCormick, B.A. (2001). Regulation of Salmonella-induced neutrophil transmigration by epithelial ADP-ribosylation factor 6. J Biol Chem *276*, 48431-48439.

Cronin, A., Decker, M., and Arand, M. (2011). Mammalian soluble epoxide hydrolase is identical to liver hepoxilin hydrolase. J Lipid Res *52*, 712-719.

Dehio, C., Gray-Owen, S.D., and Meyer, T.F. (2000). Host cell invasion by pathogenic Neisseriae. Subcell Biochem *33*, 61-96.

Dempsey, J.A., Litaker, W., Madhure, A., Snodgrass, T.L., and Cannon, J.G. (1991). Physical map of the chromosome of Neisseria gonorrhoeae FA1090 with locations of genetic markers, including opa and pil genes. J Bacteriol *173*, 5476-5486.

Dennis, E.A., and Norris, P.C. (2015). Eicosanoid storm in infection and inflammation. Nat Rev Immunol *15*, 511-523.

Dho, S., Grinstein, S., Corey, E.J., Su, W.G., and Pace-Asciak, C.R. (1990). Hepoxilin A3 induces changes in cytosolic calcium, intracellular pH and membrane potential in human neutrophils. Biochem J *266*, 63-68.

Diamant, Z., Grootendorst, D.C., Veselic-Charvat, M., Timmers, M.C., De Smet, M., Leff, J.A., Seidenberg, B.C., Zwinderman, A.H., Peszek, I., and Sterk, P.J. (1999). The effect of montelukast (MK-0476), a cysteinyl leukotriene receptor antagonist, on allergen-induced airway responses and sputum cell counts in asthma. Clin Exp Allergy *29*, 42-51.

Dietrich, M., Bartfeld, S., Munke, R., Lange, C., Ogilvie, L.A., Friedrich, A., and Meyer, T.F. (2011). Activation of NF-kappaB by Neisseria gonorrhoeae is associated with microcolony formation and type IV pilus retraction. Cellular microbiology *13*, 1168-1182.

Dixon, R.A., Diehl, R.E., Opas, E., Rands, E., Vickers, P.J., Evans, J.F., Gillard, J.W., and Miller, D.K. (1990). Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. Nature *343*, 282-284.

Dobrian, A.D., Lieb, D.C., Cole, B.K., Taylor-Fishwick, D.A., Chakrabarti, S.K., and Nadler, J.L. (2011). Functional and pathological roles of the 12- and 15-lipoxygenases. Prog Lipid Res *50*, 115-131.

Doring, Y., Soehnlein, O., and Weber, C. (2017). Neutrophil Extracellular Traps in Atherosclerosis and Atherothrombosis. Circ Res *120*, 736-743.

Douda, D.N., Grasemann, H., Pace-Asciak, C., and Palaniyar, N. (2015). A lipid mediator hepoxilin A3 is a natural inducer of neutrophil extracellular traps in human neutrophils. Mediators Inflamm *2015*, 520871.

Drake, S.L., and Koomey, M. (1995). The product of the pilQ gene is essential for the biogenesis of type IV pili in Neisseria gonorrhoeae. Mol Microbiol *18*, 975-986.

Eckmann, L., Kagnoff, M.F., and Fierer, J. (1993). Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. Infection and immunity *61*, 4569-4574.

Edwards, J.L., and Apicella, M.A. (2004). The molecular mechanisms used by Neisseria gonorrhoeae to initiate infection differ between men and women. Clin Microbiol Rev *17*, 965-981, table of contents.

Edwards, J.L., and Apicella, M.A. (2006). Neisseria gonorrhoeae PLD directly interacts with Akt kinase upon infection of primary, human, cervical epithelial cells. Cellular microbiology *8*, 1253-1271.

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

Edwards, J.L., Brown, E.J., Uk-Nham, S., Cannon, J.G., Blake, M.S., and Apicella, M.A. (2002). A co-operative interaction between Neisseria gonorrhoeae and complement receptor 3 mediates infection of primary cervical epithelial cells. Cellular microbiology *4*, 571-584.

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

Edwards, J.L., Entz, D.D., and Apicella, M.A. (2003). Gonococcal phospholipase d modulates the expression and function of complement receptor 3 in primary cervical epithelial cells. Infection and immunity *71*, 6381-6391.

Egilmez, N.K., Jong, Y.S., Mathiowitz, E., and Bankert, R.B. (2003). Tumor vaccination with cytokine-encapsulated microspheres. Methods Mol Med *75*, 687-696.

Emmendorffer, A., Hecht, M., Lohmann-Matthes, M.L., and Roesler, J. (1990). A fast and easy method to determine the production of reactive oxygen intermediates by human and murine phagocytes using dihydrorhodamine 123. J Immunol Methods *131*, 269-275.

Epp, N., Furstenberger, G., Muller, K., de Juanes, S., Leitges, M., Hausser, I., Thieme, F., Liebisch, G., Schmitz, G., and Krieg, P. (2007). 12R-lipoxygenase deficiency disrupts epidermal barrier function. J Cell Biol *177*, 173-182.

Ewald, H., Raatz, H., Boscacci, R., Furrer, H., Bucher, H.C., and Briel, M. (2015). Adjunctive corticosteroids for Pneumocystis jiroveci pneumonia in patients with HIV infection. Cochrane Database Syst Rev, CD006150.

Fabre, J.E., Goulet, J.L., Riche, E., Nguyen, M., Coggins, K., Offenbacher, S., and Koller, B.H. (2002). Transcellular biosynthesis contributes to the production of leukotrienes during inflammatory responses in vivo. J Clin Invest *109*, 1373-1380.

Fazeli, A., Bruce, C., and Anumba, D.O. (2005). Characterization of Toll-like receptors in the female reproductive tract in humans. Hum Reprod *20*, 1372-1378.

Feinen, B., Jerse, A.E., Gaffen, S.L., and Russell, M.W. (2010). Critical role of Th17 responses in a murine model of Neisseria gonorrhoeae genital infection. Mucosal Immunol *3*, 312-321.

Fichorova, R.N., and Anderson, D.J. (1999). Differential expression of immunobiological mediators by immortalized human cervical and vaginal epithelial cells. Biol Reprod *60*, 508-514.

Fichorova, R.N., Cronin, A.O., Lien, E., Anderson, D.J., and Ingalls, R.R. (2002). Response to Neisseria gonorrhoeae by cervicovaginal epithelial cells occurs in the absence of toll-like receptor 4-mediated signaling. J Immunol *168*, 2424-2432.

Fichorova, R.N., Desai, P.J., Gibson, F.C., 3rd, and Genco, C.A. (2001). Distinct proinflammatory host responses to Neisseria gonorrhoeae infection in immortalized human cervical and vaginal epithelial cells. Infection and immunity *69*, 5840-5848.

Fichorova, R.N., Rheinwald, J.G., and Anderson, D.J. (1997). Generation of papillomavirus-immortalized cell lines from normal human ectocervical, endocervical, and vaginal epithelium that maintain expression of tissue-specific differentiation proteins. Biol Reprod *57*, 847-855.

Fichorova, R.N., Zhou, F., Ratnam, V., Atanassova, V., Jiang, S., Strick, N., and Neurath, A.R. (2005). Anti-human immunodeficiency virus type 1 microbicide cellulose acetate 1,2-benzenedicarboxylate in a human in vitro model of vaginal inflammation. Antimicrob Agents Chemother *49*, 323-335.

Fisette, P.L., Ram, S., Andersen, J.M., Guo, W., and Ingalls, R.R. (2003). The Lip lipoprotein from Neisseria gonorrhoeae stimulates cytokine release and NF-kappaB activation in epithelial cells in a Toll-like receptor 2-dependent manner. J Biol Chem *278*, 46252-46260.

Fitzpatrick, F., Liggett, W., McGee, J., Bunting, S., Morton, D., and Samuelsson, B. (1984). Metabolism of leukotriene A4 by human erythrocytes. A novel cellular source of leukotriene B4. J Biol Chem *259*, 11403-11407.

Flatman, S., Hurst, J.S., McDonald-Gibson, R.G., Jonas, G.E., and Slater, T.F. (1986). Biochemical studies on a 12-lipoxygenase in human uterine cervix. Biochim Biophys Acta *883*, 7-14.

Ford-Hutchinson, A.W., Bray, M.A., Doig, M.V., Shipley, M.E., and Smith, M.J. (1980). Leukotriene B, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. Nature *286*, 264-265.

Fournier, B.M., and Parkos, C.A. (2012). The role of neutrophils during intestinal inflammation. Mucosal Immunol *5*, 354-366.

Foxman, E.F., Campbell, J.J., and Butcher, E.C. (1997). Multistep navigation and the combinatorial control of leukocyte chemotaxis. J Cell Biol *139*, 1349-1360.

Gangaiah, D., Raterman, E., Wu, H., Fortney, K.R., Gao, H., Liu, Y., Jerse, A.E., and Spinola, S.M. (2017). Both MisR (CpxR) and MisS (CpxA) are Required for Neisseria gonorrhoeae Infection in a Murine Model of Lower Genital Tract Infection. Infection and immunity.

Garcia-Verdugo, I., BenMohamed, F., Tattermusch, S., Leduc, D., Charpigny, G., Chignard, M., Ollero, M., and Touqui, L. (2012). A role for 12R-lipoxygenase in MUC5AC expression by respiratory epithelial cells. Eur Respir J *40*, 714-723.

Gaudet, R.G., and Gray-Owen, S.D. (2016). Heptose Sounds the Alarm: Innate Sensing of a Bacterial Sugar Stimulates Immunity. PLoS Pathog *12*, e1005807.

Gaudet, R.G., Guo, C.X., Molinaro, R., Kottwitz, H., Rohde, J.R., Dangeard, A.S., Arrieumerlou, C., Girardin, S.E., and Gray-Owen, S.D. (2017). Innate Recognition of Intracellular Bacterial Growth Is Driven by the TIFA-Dependent Cytosolic Surveillance Pathway. Cell Rep *19*, 1418-1430.

Gaudet, R.G., Sintsova, A., Buckwalter, C.M., Leung, N., Cochrane, A., Li, J., Cox, A.D., Moffat, J., and Gray-Owen, S.D. (2015). INNATE IMMUNITY. Cytosolic detection of the bacterial metabolite HBP activates TIFA-dependent innate immunity. Science *348*, 1251-1255.

Ginzberg, H.H., Cherapanov, V., Dong, Q., Cantin, A., McCulloch, C.A., Shannon, P.T., and Downey, G.P. (2001). Neutrophil-mediated epithelial injury during transmigration: role of elastase. Am J Physiol Gastrointest Liver Physiol *281*, G705-717.

Givan, A.L., White, H.D., Stern, J.E., Colby, E., Gosselin, E.J., Guyre, P.M., and Wira, C.R. (1997). Flow cytometric analysis of leukocytes in the human female reproductive tract: comparison of fallopian tube, uterus, cervix, and vagina. Am J Reprod Immunol *38*, 350-359.

Gotschlich, E.C., Seiff, M.E., Blake, M.S., and Koomey, M. (1987). Porin protein of Neisseria gonorrhoeae: cloning and gene structure. Proc Natl Acad Sci U S A *84*, 8135-8139.

Gray-Owen, S.D., and Blumberg, R.S. (2006). CEACAM1: contact-dependent control of immunity. Nat Rev Immunol *6*, 433-446.

Gregg, C.R., Melly, M.A., Hellerqvist, C.G., Coniglio, J.G., and McGee, Z.A. (1981). Toxic activity of purified lipopolysaccharide of Neisseria gonorrhoeae for human fallopian tube mucosa. J Infect Dis *143*, 432-439.

Grimminger, F., Menger, M., Becker, G., and Seeger, W. (1988). Potentiation of leukotriene production following sequestration of neutrophils in isolated lungs: indirect evidence for intercellular leukotriene A4 transfer. Blood *72*, 1687-1692.

Grimminger, F., Sibelius, U., and Seeger, W. (1991). Amplification of LTB4 generation in AM-PMN cocultures: transcellular 5-lipoxygenase metabolism. Am J Physiol *261*, L195-203.

Gunderson, C.W., and Seifert, H.S. (2015). Neisseria gonorrhoeae elicits extracellular traps in primary neutrophil culture while suppressing the oxidative burst. mBio *6*.

Haas, R., and Meyer, T.F. (1986). The repertoire of silent pilus genes in Neisseria gonorrhoeae: evidence for gene conversion. Cell *44*, 107-115.

Handing, J.W., and Criss, A.K. (2015). The lipooligosaccharide-modifying enzyme LptA enhances gonococcal defence against human neutrophils. Cellular microbiology *17*, 910-921.

Harizi, H., Corcuff, J.B., and Gualde, N. (2008). Arachidonic-acid-derived eicosanoids: roles in biology and immunopathology. Trends Mol Med *14*, 461-469.

Harvey, H.A., Jennings, M.P., Campbell, C.A., Williams, R., and Apicella, M.A. (2001). Receptor-mediated endocytosis of Neisseria gonorrhoeae into primary human urethral epithelial cells: the role of the asialoglycoprotein receptor. Mol Microbiol *42*, 659-672.

Harvey, H.A., Post, D.M., and Apicella, M.A. (2002). Immortalization of human urethral epithelial cells: a model for the study of the pathogenesis of and the inflammatory cytokine response to Neisseria gonorrhoeae infection. Infection and immunity *70*, 5808-5815.

Hauck, C.R., and Meyer, T.F. (2003). 'Small' talk: Opa proteins as mediators of Neisseria-host-cell communication. Curr Opin Microbiol *6*, 43-49.

Heit, B., Tavener, S., Raharjo, E., and Kubes, P. (2002). An intracellular signaling hierarchy determines direction of migration in opposing chemotactic gradients. J Cell Biol *159*, 91-102.

Hickey, D.K., Patel, M.V., Fahey, J.V., and Wira, C.R. (2011). Innate and adaptive immunity at mucosal surfaces of the female reproductive tract: stratification and integration of immune protection against the transmission of sexually transmitted infections. J Reprod Immunol *88*, 185-194.

Higashi, D.L., Lee, S.W., Snyder, A., Weyand, N.J., Bakke, A., and So, M. (2007). Dynamics of Neisseria gonorrhoeae attachment: microcolony development, cortical plaque formation, and cytoprotection. Infection and immunity *75*, 4743-4753.

Higashi, D.L., Zhang, G.H., Biais, N., Myers, L.R., Weyand, N.J., Elliott, D.A., and So, M. (2009). Influence of type IV pilus retraction on the architecture of the Neisseria gonorrhoeae-infected cell cortex. Microbiology *155*, 4084-4092.

Hitchcock, P.J. (1984). Analyses of gonococcal lipopolysaccharide in whole-cell lysates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis: stable association of lipopolysaccharide with the major outer membrane protein (protein I) of Neisseria gonorrhoeae. Infection and immunity *46*, 202-212.

Hobbs, M.M., Anderson, J.E., Balthazar, J.T., Kandler, J.L., Carlson, R.W., Ganguly, J., Begum, A.A., Duncan, J.A., Lin, J.T., Sparling, P.F.*, et al.* (2013). Lipid A's structure mediates Neisseria gonorrhoeae fitness during experimental infection of mice and men. mBio *4*, e00892-00813.

Hockenberry, A.M., Hutchens, D.M., Agellon, A., and So, M. (2016). Attenuation of the Type IV Pilus Retraction Motor Influences Neisseria gonorrhoeae Social and Infection Behavior. mBio *7*.

Hooda, Y., Shin, H.E., Bateman, T.J., and Moraes, T.F. (2017). Neisserial surface lipoproteins: structure, function and biogenesis. Pathog Dis *75*.

Hurley, B.P., Pirzai, W., Mumy, K.L., Gronert, K., and McCormick, B.A. (2011). Selective eicosanoid-generating capacity of cytoplasmic phospholipase A2 in Pseudomonas aeruginosa-infected epithelial cells. Am J Physiol Lung Cell Mol Physiol *300*, L286-294.

Hurley, B.P., Siccardi, D., Mrsny, R.J., and McCormick, B.A. (2004). Polymorphonuclear cell transmigration induced by Pseudomonas aeruginosa requires the eicosanoid hepoxilin A3. J Immunol *173*, 5712-5720.

Islam, E.A., Shaik-Dasthagirisaheb, Y., Kaushic, C., Wetzler, L.M., and Gray-Owen, S.D. (2016). The reproductive cycle is a pathogenic determinant during gonococcal pelvic inflammatory disease in mice. Mucosal Immunol *9*, 1051-1064.

Israel, E., Cohn, J., Dube, L., and Drazen, J.M. (1996). Effect of treatment with zileuton, a 5-lipoxygenase inhibitor, in patients with asthma. A randomized controlled trial. Zileuton Clinical Trial Group. JAMA *275*, 931-936.

Israel, E., Rubin, P., Kemp, J.P., Grossman, J., Pierson, W., Siegel, S.C., Tinkelman, D., Murray, J.J., Busse, W., Segal, A.T.*, et al.* (1993). The effect of inhibition of 5-lipoxygenase by zileuton in mild-to-moderate asthma. Ann Intern Med *119*, 1059-1066.

Iversen, L., Ziboh, V.A., Shimizu, T., Ohishi, N., Radmark, O., Wetterholm, A., and Kragballe, K. (1994). Identification and subcellular localization of leukotriene A4-hydrolase activity in human epidermis. J Dermatol Sci *7*, 191-201.

Jame, A.J., Lackie, P.M., Cazaly, A.M., Sayers, I., Penrose, J.F., Holgate, S.T., and Sampson, A.P. (2007). Human bronchial epithelial cells express an active and inducible biosynthetic pathway for leukotrienes B4 and C4. Clin Exp Allergy *37*, 880-892.

James, J.F., and Swanson, J. (1978). Studies on gonococcus infection. XIII. Occurrence of color/opacity colonial variants in clinical cultures. Infection and immunity *19*, 332-340.

Jean, S., Juneau, R.A., Criss, A.K., and Cornelissen, C.N. (2016). Neisseria gonorrhoeae Evades Calprotectin-Mediated Nutritional Immunity and Survives Neutrophil Extracellular Traps by Production of TdfH. Infection and immunity *84*, 2982-2994.

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 Deal, C.D. (2013). Vaccine research for gonococcal infections: where are we? Sex Transm Infect *89 Suppl 4*, iv63-68.

Jerse, A.E., Sharma, N.D., Simms, A.N., Crow, E.T., Snyder, L.A., and Shafer, W.M. (2003). A gonococcal efflux pump system enhances bacterial survival in a female mouse model of genital tract infection. Infection and immunity *71*, 5576-5582.

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

Jobard, F., Lefevre, C., Karaduman, A., Blanchet-Bardon, C., Emre, S., Weissenbach, J., Ozguc, M., Lathrop, M., Prud'homme, J.F., and Fischer, J. (2002). Lipoxygenase-3 (ALOXE3) and 12(R)-lipoxygenase (ALOX12B) are mutated in non-bullous congenital ichthyosiform erythroderma (NCIE) linked to chromosome 17p13.1. Hum Mol Genet *11*, 107-113.

John, C.M., Phillips, N.J., Stein, D.C., and Jarvis, G.A. (2017). Innate immune response to lipooligosaccharide: pivotal regulator of the pathobiology of invasive Neisseria meningitidis infections. Pathog Dis *75*.

Johnson, A.P. (1983). The pathogenic potential of commensal species of Neisseria. J Clin Pathol *36*, 213-223.

Johnson, M.B., Ball, L.M., Daily, K.P., Martin, J.N., Columbus, L., and Criss, A.K. (2015). Opa+ Neisseria gonorrhoeae exhibits reduced survival in human neutrophils via Src family kinase-mediated bacterial trafficking into mature phagolysosomes. Cellular microbiology *17*, 648-665.

Johnson, M.B., and Criss, A.K. (2011). Resistance of Neisseria gonorrhoeae to neutrophils. Front Microbiol *2*, 77.

Johnson, M.B., and Criss, A.K. (2013). Neisseria gonorrhoeae phagosomes delay fusion with primary granules to enhance bacterial survival inside human neutrophils. Cellular microbiology *15*, 1323-1340.

Juneau, R.A., Stevens, J.S., Apicella, M.A., and Criss, A.K. (2015). A thermonuclease of Neisseria gonorrhoeae enhances bacterial escape from killing by neutrophil extracellular traps. J Infect Dis *212*, 316-324.

Kandler, J.L., Holley, C.L., Reimche, J.L., Dhulipala, V., Balthazar, J.T., Muszynski, A., Carlson, R.W., and Shafer, W.M. (2016). The MisR Response Regulator Is Necessary for Intrinsic Cationic Antimicrobial Peptide and Aminoglycoside Resistance in Neisseria gonorrhoeae. Antimicrob Agents Chemother *60*, 4690-4700.

Kandler, J.L., Joseph, S.J., Balthazar, J.T., Dhulipala, V., Read, T.D., Jerse, A.E., and Shafer, W.M. (2014). Phase-variable expression of lptA modulates the resistance of Neisseria gonorrhoeae to cationic antimicrobial peptides. Antimicrob Agents Chemother *58*, 4230-4233.

Kellogg, D.S., Jr., Cohen, I.R., Norins, L.C., Schroeter, A.L., and Reising, G. (1968). Neisseria gonorrhoeae. II. Colonial variation and pathogenicity during 35 months in vitro. J Bacteriol *96*, 596-605.

Kellogg, D.S., Jr., Peacock, W.L., Jr., Deacon, W.E., Brown, L., and Pirkle, D.I. (1963). Neisseria Gonorrhoeae. I. Virulence Genetically Linked to Clonal Variation. J Bacteriol *85*, 1274-1279.

Kemp, J.P., Dockhorn, R.J., Shapiro, G.G., Nguyen, H.H., Reiss, T.F., Seidenberg, B.C., and Knorr, B. (1998). Montelukast once daily inhibits exercise-induced bronchoconstriction in 6- to 14-year-old children with asthma. J Pediatr *133*, 424-428.

Ketterer, M.R., Rice, P.A., Gulati, S., Kiel, S., Byerly, L., Fortenberry, J.D., Soper, D.E., and Apicella, M.A. (2016). Desialylation of Neisseria gonorrhoeae Lipooligosaccharide by Cervicovaginal Microbiome Sialidases: The Potential for Enhancing Infectivity in Men. J Infect Dis *214*, 1621-1628.

Kim, J.J., Zhou, D., Mandrell, R.E., and Griffiss, J.M. (1992). Effect of exogenous sialylation of the lipooligosaccharide of Neisseria gonorrhoeae on opsonophagocytosis. Infection and immunity *60*, 4439-4442.

Kirchner, M., Heuer, D., and Meyer, T.F. (2005). CD46-independent binding of neisserial type IV pili and the major pilus adhesin, PilC, to human epithelial cells. Infection and immunity *73*, 3072-3082.

Kirchner, M., and Meyer, T.F. (2005). The PilC adhesin of the Neisseria type IV pilus-binding specificities and new insights into the nature of the host cell receptor. Mol Microbiol *56*, 945-957.

Knilans, K.J., Hackett, K.T., Anderson, J.E., Weng, C., Dillard, J.P., and Duncan, J.A. (2017). Neisseria gonorrhoeae Lytic Transglycosylases LtgA and LtgD Reduce Host Innate Immune Signaling through TLR2 and NOD2. ACS Infect Dis.

Kohler, H., Rodrigues, S.P., and McCormick, B.A. (2002). Shigella flexneri Interactions with the Basolateral Membrane Domain of Polarized Model Intestinal Epithelium: Role of Lipopolysaccharide in Cell Invasion and in Activation of the Mitogen-Activated Protein Kinase ERK. Infection and immunity *70*, 1150-1158.

Kretz, M., Siprashvili, Z., Chu, C., Webster, D.E., Zehnder, A., Qu, K., Lee, C.S., Flockhart, R.J., Groff, A.F., Chow, J.*, et al.* (2013). Control of somatic tissue differentiation by the long non-coding RNA TINCR. Nature *493*, 231-235.

Krieg, P., Rosenberger, S., de Juanes, S., Latzko, S., Hou, J., Dick, A., Kloz, U., van der Hoeven, F., Hausser, I., Esposito, I.*, et al.* (2013). Aloxe3 knockout mice reveal a function of epidermal lipoxygenase-3 as hepoxilin synthase and its pivotal role in barrier formation. J Invest Dermatol *133*, 172-180.

Kruger, P., Saffarzadeh, M., Weber, A.N., Rieber, N., Radsak, M., von Bernuth, H., Benarafa, C., Roos, D., Skokowa, J., and Hartl, D. (2015). Neutrophils: Between host defence, immune modulation, and tissue injury. PLoS Pathog *11*, e1004651.

Kucharzik, T., Hudson, J.T., 3rd, Lugering, A., Abbas, J.A., Bettini, M., Lake, J.G., Evans, M.E., Ziegler, T.R., Merlin, D., Madara, J.L.*, et al.* (2005). Acute induction of human IL-8 production by intestinal epithelium triggers neutrophil infiltration without mucosal injury. Gut *54*, 1565-1572.

Kucharzik, T., Walsh, S.V., Chen, J., Parkos, C.A., and Nusrat, A. (2001). Neutrophil transmigration in inflammatory bowel disease is associated with differential expression of epithelial intercellular junction proteins. Am J Pathol *159*, 2001-2009.

Kupsch, E.M., Knepper, B., Kuroki, T., Heuer, I., and Meyer, T.F. (1993). Variable opacity (Opa) outer membrane proteins account for the cell tropisms displayed by Neisseria gonorrhoeae for human leukocytes and epithelial cells. EMBO J *12*, 641-650.

Lammermann, T., Afonso, P.V., Angermann, B.R., Wang, J.M., Kastenmuller, W., Parent, C.A., and Germain, R.N. (2013). Neutrophil swarms require LTB4 and integrins at sites of cell death in vivo. Nature *498*, 371-375.

Laniewski, P., Gomez, A., Hire, G., So, M., and Herbst-Kralovetz, M.M. (2017). Human Three-Dimensional Endometrial Epithelial Cell Model To Study Host Interactions with Vaginal Bacteria and Neisseria gonorrhoeae. Infection and immunity *85*.

Lawrence, D.W., Bruyninckx, W.J., Louis, N.A., Lublin, D.M., Stahl, G.L., Parkos, C.A., and Colgan, S.P. (2003). Antiadhesive role of apical decay-accelerating factor (CD55) in human neutrophil transmigration across mucosal epithelia. J Exp Med *198*, 999-1010.

Lazarus, S.C., Wong, H.H., Watts, M.J., Boushey, H.A., Lavins, B.J., and Minkwitz, M.C. (1997). The leukotriene receptor antagonist zafirlukast inhibits sulfur dioxide-induced bronchoconstriction in patients with asthma. Am J Respir Crit Care Med *156*, 1725-1730.

Le Bel, M., Brunet, A., and Gosselin, J. (2014). Leukotriene B4, an endogenous stimulator of the innate immune response against pathogens. J Innate Immun *6*, 159-168.

Lee, C.A., Silva, M., Siber, A.M., Kelly, A.J., Galyov, E., and McCormick, B.A. (2000). A secreted Salmonella protein induces a proinflammatory response in epithelial cells, which promotes neutrophil migration. Proc Natl Acad Sci U S A *97*, 12283-12288.

Lee, S.W., Higashi, D.L., Snyder, A., Merz, A.J., Potter, L., and So, M. (2005). PilT is required for PI(3,4,5)P3-mediated crosstalk between Neisseria gonorrhoeae and epithelial cells. Cellular microbiology *7*, 1271-1284.

Lee, W., Su Kim, H., and Lee, G.R. (2015). Leukotrienes induce the migration of Th17 cells. Immunol Cell Biol *93*, 472-479.

Leuzzi, R., Serino, L., Scarselli, M., Savino, S., Fontana, M.R., Monaci, E., Taddei, A., Fischer, G., Rappuoli, R., and Pizza, M. (2005). Ng-MIP, a surface-exposed lipoprotein of Neisseria gonorrhoeae, has a peptidyl-prolyl cis/trans isomerase (PPIase) activity and is involved in persistence in macrophages. Mol Microbiol *58*, 669-681.

Li, P., Oh, D.Y., Bandyopadhyay, G., Lagakos, W.S., Talukdar, S., Osborn, O., Johnson, A., Chung, H., Maris, M., Ofrecio, J.M.*, et al.* (2015). LTB4 promotes insulin resistance in obese mice by acting on macrophages, hepatocytes and myocytes. Nat Med *21*, 239-247.

Liston, T.E., Conklyn, M.J., Houser, J., Wilner, K.D., Johnson, A., Apseloff, G., Whitacre, C., and Showell, H.J. (1998). Pharmacokinetics and pharmacodynamics of the leukotriene B4 receptor antagonist CP-105,696 in man following single oral administration. Br J Clin Pharmacol *45*, 115-121.

Liu, G., Tang, C.M., and Exley, R.M. (2015). Non-pathogenic Neisseria: members of an abundant, multi-habitat, diverse genus. Microbiology *161*, 1297-1312.

Liu, Y., Buhring, H.J., Zen, K., Burst, S.L., Schnell, F.J., Williams, I.R., and Parkos, C.A. (2002). Signal regulatory protein (SIRPalpha), a cellular ligand for CD47, regulates neutrophil transmigration. J Biol Chem *277*, 10028-10036.

Liu, Y., Feinen, B., and Russell, M.W. (2011). New concepts in immunity to Neisseria gonorrhoeae: innate responses and suppression of adaptive immunity favor the pathogen, not the host. Front Microbiol *2*, 52.

Liu, Y., Hammer, L.A., Liu, W., Hobbs, M.M., Zielke, R.A., Sikora, A.E., Jerse, A.E., Egilmez, N.K., and Russell, M.W. (2017). Experimental vaccine induces Th1-driven immune responses and resistance to Neisseria gonorrhoeae infection in a murine model. Mucosal Immunol.

Liu, Y., Islam, E.A., Jarvis, G.A., Gray-Owen, S.D., and Russell, M.W. (2012). Neisseria gonorrhoeae selectively suppresses the development of Th1 and Th2 cells, and enhances Th17 cell responses, through TGF-beta-dependent mechanisms. Mucosal Immunol *5*, 320-331.

Liu, Y., Liu, W., and Russell, M.W. (2014). Suppression of host adaptive immune responses by Neisseria gonorrhoeae: role of interleukin 10 and type 1 regulatory T cells. Mucosal Immunol *7*, 165-176.

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

Liu, Y., and Russell, M.W. (2011). Diversion of the immune response to Neisseria gonorrhoeae from Th17 to Th1/Th2 by treatment with anti-transforming growth factor beta antibody generates immunological memory and protective immunity. mBio *2*, e00095-00011.

Liu, Y., Shaw, S.K., Ma, S., Yang, L., Luscinskas, F.W., and Parkos, C.A. (2004). Regulation of leukocyte transmigration: cell surface interactions and signaling events. J Immunol *172*, 7-13.

Lorenzen, D.R., Gunther, D., Pandit, J., Rudel, T., Brandt, E., and Meyer, T.F. (2000). Neisseria gonorrhoeae porin modifies the oxidative burst of human professional phagocytes. Infection and immunity *68*, 6215-6222.

Magalhaes, J.G., Sorbara, M.T., Girardin, S.E., and Philpott, D.J. (2011). What is new with Nods? Curr Opin Immunol *23*, 29-34.

Majumdar, R., Tavakoli Tameh, A., and Parent, C.A. (2016). Exosomes Mediate LTB4 Release during Neutrophil Chemotaxis. PLoS Biol *14*, e1002336.

Maloy, K.J., and Powrie, F. (2011). Intestinal homeostasis and its breakdown in inflammatory bowel disease. Nature *474*, 298-306.

Martin, R., Soberon, N., Vaneechoutte, M., Florez, A.B., Vazquez, F., and Suarez, J.E. (2008). Characterization of indigenous vaginal lactobacilli from healthy women as probiotic candidates. Int Microbiol *11*, 261-266.

Mashima, R., and Okuyama, T. (2015). The role of lipoxygenases in pathophysiology; new insights and future perspectives. Redox Biol *6*, 297-310.

Maskrey, B.H., Bermudez-Fajardo, A., Morgan, A.H., Stewart-Jones, E., Dioszeghy, V., Taylor, G.W., Baker, P.R., Coles, B., Coffey, M.J., Kuhn, H.*, et al.* (2007). Activated platelets and monocytes generate four hydroxyphosphatidylethanolamines via lipoxygenase. J Biol Chem *282*, 20151-20163.

Massari, P., Henneke, P., Ho, Y., Latz, E., Golenbock, D.T., and Wetzler, L.M. (2002). Cutting edge: Immune stimulation by neisserial porins is toll-like receptor 2 and MyD88 dependent. J Immunol *168*, 1533-1537.

Massari, P., Ho, Y., and Wetzler, L.M. (2000). Neisseria meningitidis porin PorB interacts with mitochondria and protects cells from apoptosis. Proc Natl Acad Sci U S A *97*, 9070-9075.

Masson, L., Mlisana, K., Little, F., Werner, L., Mkhize, N.N., Ronacher, K., Gamieldien, H., Williamson, C., McKinnon, L.R., Walzl, G.*, et al.* (2014). Defining genital tract cytokine signatures of sexually transmitted infections and bacterial vaginosis in women at high risk of HIV infection: a cross-sectional study. Sex Transm Infect *90*, 580-587.

Masson, L., Salkinder, A.L., Olivier, A.J., McKinnon, L.R., Gamieldien, H., Mlisana, K., Scriba, T.J., Lewis, D.A., Little, F., Jaspan, H.B.*, et al.* (2015). Relationship between female genital tract infections, mucosal interleukin-17 production and local T helper type 17 cells. Immunology *146*, 557-567.

Mavrogiorgos, N., Mekasha, S., Yang, Y., Kelliher, M.A., and Ingalls, R.R. (2014). Activation of NOD receptors by Neisseria gonorrhoeae modulates the innate immune response. Innate Immun *20*, 377-389.

Mayor, M.T., Roett, M.A., and Uduhiri, K.A. (2012). Diagnosis and management of gonococcal infections. Am Fam Physician *86*, 931-938.

McClure, R., and Massari, P. (2014). TLR-Dependent Human Mucosal Epithelial Cell Responses to Microbial Pathogens. Front Immunol *5*, 386.

McCormick, B.A., Hofman, P.M., Kim, J., Carnes, D.K., Miller, S.I., and Madara, J.L. (1995). Surface attachment of Salmonella typhimurium to intestinal epithelia imprints the subepithelial matrix with gradients chemotactic for neutrophils. J Cell Biol *131*, 1599-1608.

McCormick, B.A., Parkos, C.A., Colgan, S.P., Carnes, D.K., and Madara, J.L. (1998). Apical secretion of a pathogen-elicited epithelial chemoattractant activity in response to surface colonization of intestinal epithelia by Salmonella typhimurium. J Immunol *160*, 455-466.

McGee, J.E., and Fitzpatrick, F.A. (1986). Erythrocyte-neutrophil interactions: formation of leukotriene B4 by transcellular biosynthesis. Proc Natl Acad Sci U S A *83*, 1349-1353.

McGee, Z.A., Jensen, R.L., Clemens, C.M., Taylor-Robinson, D., Johnson, A.P., and Gregg, C.R. (1999). Gonococcal infection of human fallopian tube mucosa in organ culture: relationship of mucosal tissue TNF-alpha concentration to sloughing of ciliated cells. Sex Transm Dis *26*, 160-165.

Melly, M.A., Gregg, C.R., and McGee, Z.A. (1981). Studies of toxicity of Neisseria gonorrhoeae for human fallopian tube mucosa. J Infect Dis *143*, 423-431.

Melly, M.A., McGee, Z.A., and Rosenthal, R.S. (1984). Ability of monomeric peptidoglycan fragments from Neisseria gonorrhoeae to damage human fallopian-tube mucosa. J Infect Dis *149*, 378-386.

Mertens, G., Van der Schueren, B., van den Berghe, H., and David, G. (1996). Heparan sulfate expression in polarized epithelial cells: the apical sorting of glypican (GPI-anchored proteoglycan) is inversely related to its heparan sulfate content. J Cell Biol *132*, 487-497.

Merz, A.J., Enns, C.A., and So, M. (1999). Type IV pili of pathogenic Neisseriae elicit cortical plaque formation in epithelial cells. Mol Microbiol *32*, 1316-1332.

Merz, A.J., and So, M. (1997). Attachment of piliated, Opa- and Opc- gonococci and meningococci to epithelial cells elicits cortical actin rearrangements and clustering of tyrosine-phosphorylated proteins. Infection and immunity *65*, 4341-4349.

Merz, A.J., and So, M. (2000). Interactions of pathogenic neisseriae with epithelial cell membranes. Annu Rev Cell Dev Biol *16*, 423-457.

Merz, A.J., So, M., and Sheetz, M.P. (2000). Pilus retraction powers bacterial twitching motility. Nature *407*, 98-102.

Mittal, M., Siddiqui, M.R., Tran, K., Reddy, S.P., and Malik, A.B. (2014). Reactive oxygen species in inflammation and tissue injury. Antioxid Redox Signal *20*, 1126-1167.

Morand, P.C., Bille, E., Morelle, S., Eugene, E., Beretti, J.L., Wolfgang, M., Meyer, T.F., Koomey, M., and Nassif, X. (2004). Type IV pilus retraction in pathogenic Neisseria is regulated by the PilC proteins. EMBO J *23*, 2009-2017.

Morgan, A.H., Dioszeghy, V., Maskrey, B.H., Thomas, C.P., Clark, S.R., Mathie, S.A., Lloyd, C.M., Kuhn, H., Topley, N., Coles, B.C.*, et al.* (2009). Phosphatidylethanolamine-esterified eicosanoids in the mouse: tissue localization and inflammation-dependent formation in Th-2 disease. J Biol Chem *284*, 21185-21191.

Morgan, A.H., Hammond, V.J., Morgan, L., Thomas, C.P., Tallman, K.A., Garcia-Diaz, Y.R., McGuigan, C., Serpi, M., Porter, N.A., Murphy, R.C.*, et al.* (2010a). Quantitative assays for esterified oxylipins generated by immune cells. Nat Protoc *5*, 1919-1931.

Morgan, L.T., Thomas, C.P., Kuhn, H., and O'Donnell, V.B. (2010b). Thrombin-activated human platelets acutely generate oxidized docosahexaenoic-acid-containing phospholipids via 12-lipoxygenase. Biochem J *431*, 141-148.

Morisseau, C., and Hammock, B.D. (2007). Measurement of soluble epoxide hydrolase (sEH) activity. Curr Protoc Toxicol *Chapter 4*, Unit 4 23.

Morisseau, C., and Hammock, B.D. (2013). Impact of soluble epoxide hydrolase and epoxyeicosanoids on human health. Annu Rev Pharmacol Toxicol *53*, 37-58.

Morisseau, C., Schebb, N.H., Dong, H., Ulu, A., Aronov, P.A., and Hammock, B.D. (2012). Role of soluble epoxide hydrolase phosphatase activity in the metabolism of lysophosphatidic acids. Biochem Biophys Res Commun *419*, 796-800.

Mrsny, R.J., Gewirtz, A.T., Siccardi, D., Savidge, T., Hurley, B.P., Madara, J.L., and McCormick, B.A. (2004). Identification of hepoxilin A3 in inflammatory events: a required role in neutrophil migration across intestinal epithelia. Proc Natl Acad Sci U S A *101*, 7421-7426.

Muenzner, P., Billker, O., Meyer, T.F., and Naumann, M. (2002). Nuclear factor-kappa B directs carcinoembryonic antigen-related cellular adhesion molecule 1 receptor expression in Neisseria gonorrhoeae-infected epithelial cells. J Biol Chem *277*, 7438-7446.

Muller, A., Gunther, D., Brinkmann, V., Hurwitz, R., Meyer, T.F., and Rudel, T. (2000). Targeting of the pro-apoptotic VDAC-like porin (PorB) of Neisseria gonorrhoeae to mitochondria of infected cells. EMBO J *19*, 5332-5343.

Muller, A., Gunther, D., Dux, F., Naumann, M., Meyer, T.F., and Rudel, T. (1999). Neisserial porin (PorB) causes rapid calcium influx in target cells and induces apoptosis by the activation of cysteine proteases. EMBO J *18*, 339-352.

Muller, W.A. (2003). Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. Trends Immunol *24*, 327-334.

Mumy, K.L., Bien, J.D., Pazos, M.A., Gronert, K., Hurley, B.P., and McCormick, B.A. (2008). Distinct isoforms of phospholipase A2 mediate the ability of Salmonella enterica serotype typhimurium and Shigella flexneri to induce the transepithelial migration of neutrophils. Infection and immunity *76*, 3614-3627.

Murakami, M., and Kudo, I. (2002). Phospholipase A2. J Biochem *131*, 285-292.

Murphy, G.L., Connell, T.D., Barritt, D.S., Koomey, M., and Cannon, J.G. (1989). Phase variation of gonococcal protein II: regulation of gene expression by slipped-strand mispairing of a repetitive DNA sequence. Cell *56*, 539-547.

Nadeau, W.J., Pistole, T.G., and McCormick, B.A. (2002). Polymorphonuclear leukocyte migration across model intestinal epithelia enhances Salmonella typhimurium killing via the epithelial derived cytokine, IL-6. Microbes Infect *4*, 1379-1387.

Nash, S., Stafford, J., and Madara, J.L. (1987). Effects of polymorphonuclear leukocyte transmigration on the barrier function of cultured intestinal epithelial monolayers. J Clin Invest *80*, 1104-1113.

Nash, S., Stafford, J., and Madara, J.L. (1988). The selective and superoxide-independent disruption of intestinal epithelial tight junctions during leukocyte transmigration. Lab Invest *59*, 531-537.

Naumann, M., Rudel, T., Wieland, B., Bartsch, C., and Meyer, T.F. (1998). Coordinate activation of activator protein 1 and inflammatory cytokines in response to Neisseria gonorrhoeae epithelial cell contact involves stress response kinases. J Exp Med *188*, 1277-1286.

Naumann, M., Wessler, S., Bartsch, C., Wieland, B., and Meyer, T.F. (1997). Neisseria gonorrhoeae epithelial cell interaction leads to the activation of the transcription factors nuclear factor kappaB and activator protein 1 and the induction of inflammatory cytokines. J Exp Med *186*, 247-258.

Nelson, H., Kemp, J., Berger, W., Corren, J., Casale, T., Dube, L., Walton-Bowen, K., LaVallee, N., and Stepanians, M. (2007). Efficacy of zileuton controlled-release tablets administered twice daily in the treatment of moderate persistent asthma: a 3-month randomized controlled study. Ann Allergy Asthma Immunol *99*, 178-184.

Nemeth, T., and Mocsai, A. (2016). Feedback Amplification of Neutrophil Function. Trends Immunol *37*, 412-424.

Nusrat, A., Parkos, C.A., Liang, T.W., Carnes, D.K., and Madara, J.L. (1997). Neutrophil migration across model intestinal epithelia: monolayer disruption and subsequent events in epithelial repair. Gastroenterology *113*, 1489-1500.

Obergfell, K.P., and Seifert, H.S. (2015). Mobile DNA in the Pathogenic Neisseria. Microbiol Spectr *3*.

Ortiz, M.C., Lefimil, C., Rodas, P.I., Vernal, R., Lopez, M., Acuna-Castillo, C., Imarai, M., and Escobar, A. (2015). Neisseria gonorrhoeae Modulates Immunity by Polarizing Human Macrophages to a M2 Profile. PLoS One *10*, e0130713.

Pace-Asciak, C.R. (2015). Pathophysiology of the hepoxilins. Biochim Biophys Acta *1851*, 383-396.

Packiam, M., Yedery, R.D., Begum, A.A., Carlson, R.W., Ganguly, J., Sempowski, G.D., Ventevogel, M.S., Shafer, W.M., and Jerse, A.E. (2014). Phosphoethanolamine decoration of Neisseria gonorrhoeae lipid A plays a dual immunostimulatory and protective role during experimental genital tract infection. Infection and immunity *82*, 2170-2179.

Papayannopoulos, V., and Zychlinsky, A. (2009). NETs: a new strategy for using old weapons. Trends Immunol *30*, 513-521.

Parkos, C.A., Colgan, S.P., Liang, T.W., Nusrat, A., Bacarra, A.E., Carnes, D.K., and Madara, J.L. (1996). CD47 mediates post-adhesive events required for neutrophil migration across polarized intestinal epithelia. J Cell Biol *132*, 437-450.

Parkos, C.A., Delp, C., Arnaout, M.A., and Madara, J.L. (1991). Neutrophil migration across a cultured intestinal epithelium. Dependence on a CD11b/CD18-mediated event and enhanced efficiency in physiological direction. J Clin Invest *88*, 1605-1612.

Pazos, M., Siccardi, D., Mumy, K.L., Bien, J.D., Louie, S., Shi, H.N., Gronert, K., Mrsny, R.J., and McCormick, B.A. (2008). Multidrug resistance-associated transporter 2 regulates mucosal inflammation by facilitating the synthesis of hepoxilin A3. J Immunol *181*, 8044-8052.

Pazos, M.A., Lanter, B.B., Yonker, L.M., Eaton, A.D., Pirzai, W., Gronert, K., Bonventre, J.V., and Hurley, B.P. (2017). Pseudomonas aeruginosa ExoU augments neutrophil transepithelial migration. PLoS Pathog *13*, e1006548.

Pazos, M.A., Pirzai, W., Yonker, L.M., Morisseau, C., Gronert, K., and Hurley, B.P. (2015). Distinct cellular sources of hepoxilin A3 and leukotriene B4 are used to coordinate bacterial-induced neutrophil transepithelial migration. J Immunol *194*, 1304-1315.

Pearlman, D.S., Ostrom, N.K., Bronsky, E.A., Bonuccelli, C.M., and Hanby, L.A. (1999). The leukotriene D4-receptor antagonist zafirlukast attenuates exercise-induced bronchoconstriction in children. J Pediatr *134*, 273-279.

Pelletier, M., Maggi, L., Micheletti, A., Lazzeri, E., Tamassia, N., Costantini, C., Cosmi, L., Lunardi, C., Annunziato, F., Romagnani, S*., et al.* (2010). Evidence for a cross-talk between human neutrophils and Th17 cells. Blood *115*, 335-343.

Pham, C.T. (2006). Neutrophil serine proteases: specific regulators of inflammation. Nat Rev Immunol *6*, 541-550.

Phillips, R.M., Six, D.A., Dennis, E.A., and Ghosh, P. (2003). In vivo phospholipase activity of the Pseudomonas aeruginosa cytotoxin ExoU and protection of mammalian cells with phospholipase A2 inhibitors. J Biol Chem *278*, 41326-41332.

Porto, B.N., and Stein, R.T. (2016). Neutrophil Extracellular Traps in Pulmonary Diseases: Too Much of a Good Thing? Front Immunol *7*, 311.

Qiu, H., Johansson, A.S., Sjostrom, M., Wan, M., Schroder, O., Palmblad, J., and Haeggstrom, J.Z. (2006). Differential induction of BLT receptor expression on human endothelial cells by lipopolysaccharide, cytokines, and leukotriene B4. Proc Natl Acad Sci U S A *103*, 6913-6918.

Quayle, A.J. (2002). The innate and early immune response to pathogen challenge in the female genital tract and the pivotal role of epithelial cells. J Reprod Immunol *57*, 61-79.

Radmark, O., Funk, C.D., Fu, J.Y., Matsumoto, T., Jornvall, H., Shimizu, T., Medina, J.F., Haeggstrom, J., Kumlin, M., and Samuelsson, B. (1989). Leukotriene A4-hydrolase, cloning of the human enzyme and tissue distribution in the rat. Adv Prostaglandin Thromboxane Leukot Res *19*, 484-487.

Ragland, S.A., and Criss, A.K. (2017). From bacterial killing to immune modulation: Recent insights into the functions of lysozyme. PLoS Pathog *13*, e1006512.

Ragland, S.A., Schaub, R.E., Hackett, K.T., Dillard, J.P., and Criss, A.K. (2017). Two lytic transglycosylases in Neisseria gonorrhoeae impart resistance to killing by lysozyme and human neutrophils. Cellular microbiology *19*.

Rahman, M., Kallstrom, H., Normark, S., and Jonsson, A.B. (1997). PilC of pathogenic Neisseria is associated with the bacterial cell surface. Mol Microbiol *25*, 11-25.

Ramsey, K.H., Schneider, H., Cross, A.S., Boslego, J.W., Hoover, D.L., Staley, T.L., Kuschner, R.A., and Deal, C.D. (1995). Inflammatory cytokines produced in response to experimental human gonorrhea. J Infect Dis *172*, 186-191.

Reid, G.K., Kargman, S., Vickers, P.J., Mancini, J.A., Leveille, C., Ethier, D., Miller, D.K., Gillard, J.W., Dixon, R.A., and Evans, J.F. (1990). Correlation between expression of 5-

lipoxygenase-activating protein, 5-lipoxygenase, and cellular leukotriene synthesis. J Biol Chem *265*, 19818-19823.

Reimer, A., Seufert, F., Weiwad, M., Ebert, J., Bzdyl, N.M., Kahler, C.M., Sarkar-Tyson, M., Holzgrabe, U., Rudel, T., and Kozjak-Pavlovic, V. (2016). Inhibitors of macrophage infectivity potentiator-like PPIases affect neisserial and chlamydial pathogenicity. Int J Antimicrob Agents *48*, 401-408.

Rest, R.F., and Frangipane, J.V. (1992). Growth of Neisseria gonorrhoeae in CMP-N-acetylneuraminic acid inhibits nonopsonic (opacity-associated outer membrane protein-mediated) interactions with human neutrophils. Infection and immunity *60*, 989-997.

Reynaud, D., Demin, P., and Pace-Asciak, C.R. (1995a). Hepoxilin binding in human neutrophils. Biochem Biophys Res Commun *207*, 191-194.

Reynaud, D., Demin, P.M., and Pace-Asciak, C.R. (1995b). Coupling of hepoxilin A3-specific binding with calcium-mobilizing actions in human neutrophils. Agents Actions Suppl *45*, 291-296.

Rodas, P.I., Perez, D., Jaffret, C., Gonzalez, Y., Carreno, C., Tapia, C.V., Osorio, E., Velasquez, L.A., and Christodoulides, M. (2016). Modified profile of matrix metalloproteinase-2 and -9 production by human Fallopian tube epithelial cells following infection in vitro with Neisseria gonorrhoeae. J Infect Dis.

Rouzer, C.A., Matsumoto, T., and Samuelsson, B. (1986). Single protein from human leukocytes possesses 5-lipoxygenase and leukotriene A4 synthase activities. Proc Natl Acad Sci U S A *83*, 857-861.

Rudel, T., Scheurerpflug, I., and Meyer, T.F. (1995). Neisseria PilC protein identified as type-4 pilus tip-located adhesin. Nature *373*, 357-359.

Rudel, T., Schmid, A., Benz, R., Kolb, H.A., Lang, F., and Meyer, T.F. (1996). Modulation of Neisseria porin (PorB) by cytosolic ATP/GTP of target cells: parallels between pathogen accommodation and mitochondrial endosymbiosis. Cell *85*, 391-402.

Rudel, T., van Putten, J.P., Gibbs, C.P., Haas, R., and Meyer, T.F. (1992). Interaction of two variable proteins (PilE and PilC) required for pilus-mediated adherence of Neisseria gonorrhoeae to human epithelial cells. Mol Microbiol *6*, 3439-3450.

Sabbione, F., Keitelman, I.A., Iula, L., Ferrero, M., Giordano, M.N., Baldi, P., Rumbo, M., Jancic, C., and Trevani, A.S. (2017). Neutrophil Extracellular Traps Stimulate Proinflammatory Responses in Human Airway Epithelial Cells. J Innate Immun.

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

Sadik, C.D., and Luster, A.D. (2012). Lipid-cytokine-chemokine cascades orchestrate leukocyte recruitment in inflammation. J Leukoc Biol *91*, 207-215.

Saffarzadeh, M., Juenemann, C., Queisser, M.A., Lochnit, G., Barreto, G., Galuska, S.P., Lohmeyer, J., and Preissner, K.T. (2012). Neutrophil extracellular traps directly induce epithelial and endothelial cell death: a predominant role of histones. PLoS One *7*, e32366.

Sala, A., Bolla, M., Zarini, S., Muller-Peddinghaus, R., and Folco, G. (1996). Release of leukotriene A4 versus leukotriene B4 from human polymorphonuclear leukocytes. J Biol Chem *271*, 17944-17948.

Saliba, A.M., Nascimento, D.O., Silva, M.C., Assis, M.C., Gayer, C.R., Raymond, B., Coelho, M.G., Marques, E.A., Touqui, L., Albano, R.M*., et al.* (2005). Eicosanoid-mediated proinflammatory activity of Pseudomonas aeruginosa ExoU. Cellular microbiology *7*, 1811-1822.

Samuelsson, B., and Funk, C.D. (1989). Enzymes involved in the biosynthesis of leukotriene B4. J Biol Chem *264*, 19469-19472.

Sarantis, H., and Gray-Owen, S.D. (2007). The specific innate immune receptor CEACAM3 triggers neutrophil bactericidal activities via a Syk kinase-dependent pathway. Cellular microbiology *9*, 2167-2180.

Sarantis, H., and Gray-Owen, S.D. (2012). Defining the roles of human carcinoembryonic antigen-related cellular adhesion molecules during neutrophil responses to Neisseria gonorrhoeae. Infection and immunity *80*, 345-358.

Sathe, A., and Reddy, K.V. (2014). TLR9 and RIG-I signaling in human endocervical epithelial cells modulates inflammatory responses of macrophages and dendritic cells in vitro. PLoS One *9*, e83882.

Sato, H., Frank, D.W., Hillard, C.J., Feix, J.B., Pankhaniya, R.R., Moriyama, K., Finck-Barbancon, V., Buchaklian, A., Lei, M., Long, R.M.*, et al.* (2003). The mechanism of action of the Pseudomonas aeruginosa-encoded type III cytotoxin, ExoU. EMBO J *22*, 2959-2969.

Seib, K.L., Simons, M.P., Wu, H.J., McEwan, A.G., Nauseef, W.M., Apicella, M.A., and Jennings, M.P. (2005). Investigation of oxidative stress defenses of Neisseria gonorrhoeae by using a human polymorphonuclear leukocyte survival assay. Infection and immunity *73*, 5269-5272.

Seib, K.L., Tseng, H.J., McEwan, A.G., Apicella, M.A., and Jennings, M.P. (2004). Defenses against oxidative stress in Neisseria gonorrhoeae and Neisseria meningitidis: distinctive systems for different lifestyles. J Infect Dis *190*, 136-147.

Serhan, C.N. (2014). Pro-resolving lipid mediators are leads for resolution physiology. Nature *510*, 92-101.

Silva, M., Song, C., Nadeau, W.J., Matthews, J.B., and McCormick, B.A. (2004). Salmonella typhimurium SipA-induced neutrophil transepithelial migration: involvement of a PKC-alpha-dependent signal transduction pathway. Am J Physiol Gastrointest Liver Physiol *286*, G1024-1031.

Simons, M.P., Nauseef, W.M., and Apicella, M.A. (2005). Interactions of Neisseria gonorrhoeae with adherent polymorphonuclear leukocytes. Infection and immunity *73*, 1971-1977.

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

Sintsova, A., Wong, H., MacDonald, K.S., Kaul, R., Virji, M., and Gray-Owen, S.D. (2015). Selection for a CEACAM receptor-specific binding phenotype during Neisseria gonorrhoeae infection of the human genital tract. Infection and immunity *83*, 1372-1383.

Sitaraman, S.V., Merlin, D., Wang, L., Wong, M., Gewirtz, A.T., Si-Tahar, M., and Madara, J.L. (2001). Neutrophil-epithelial crosstalk at the intestinal lumenal surface mediated by reciprocal secretion of adenosine and IL-6. J Clin Invest *107*, 861-869.

Smirnov, A., Daily, K.P., and Criss, A.K. (2014). Assembly of NADPH oxidase in human neutrophils is modulated by the opacity-associated protein expression State of Neisseria gonorrhoeae. Infection and immunity *82*, 1036-1044.

Smirnov, A., Solga, M.D., Lannigan, J., and Criss, A.K. (2015). An improved method for differentiating cell-bound from internalized particles by imaging flow cytometry. J Immunol Methods *423*, 60-69.

Snapper, C.M., Rosas, F.R., Kehry, M.R., Mond, J.J., and Wetzler, L.M. (1997). Neisserial porins may provide critical second signals to polysaccharide-activated murine B cells for induction of immunoglobulin secretion. Infection and immunity *65*, 3203-3208.

Snelgrove, R.J., Jackson, P.L., Hardison, M.T., Noerager, B.D., Kinloch, A., Gaggar, A., Shastry, S., Rowe, S.M., Shim, Y.M., Hussell, T.*, et al.* (2010). A critical role for LTA4H in limiting chronic pulmonary neutrophilic inflammation. Science *330*, 90-94.

Soler-Garcia, A.A., and Jerse, A.E. (2004). A Neisseria gonorrhoeae catalase mutant is more sensitive to hydrogen peroxide and paraquat, an inducer of toxic oxygen radicals. Microb Pathog *37*, 55-63.

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

Song, W., Ma, L., Chen, R., and Stein, D.C. (2000). Role of lipooligosaccharide in Opa-independent invasion of Neisseria gonorrhoeae into human epithelial cells. J Exp Med *191*, 949-960.

Stern, A., Brown, M., Nickel, P., and Meyer, T.F. (1986). Opacity genes in Neisseria gonorrhoeae: control of phase and antigenic variation. Cell *47*, 61-71.

Stevens, J.S., and Criss, A.K. (2018). Pathogenesis of Neisseria gonorrhoeae in the female reproductive tract: neutrophilic host response, sustained infection, and clinical sequelae. Curr Opin Hematol *25*, 13-21.

Stohl, E.A., Chan, Y.A., Hackett, K.T., Kohler, P.L., Dillard, J.P., and Seifert, H.S. (2012). Neisseria gonorrhoeae virulence factor NG1686 is a bifunctional M23B family metallopeptidase that influences resistance to hydrogen peroxide and colony morphology. J Biol Chem *287*, 11222-11233.

Stohl, E.A., Criss, A.K., and Seifert, H.S. (2005). The transcriptome response of Neisseria gonorrhoeae to hydrogen peroxide reveals genes with previously uncharacterized roles in oxidative damage protection. Mol Microbiol *58*, 520-532.

Stohl, E.A., Dale, E.M., Criss, A.K., and Seifert, H.S. (2013). Neisseria gonorrhoeae metalloprotease NGO1686 is required for full piliation, and piliation is required for resistance to H2O2- and neutrophil-mediated killing. mBio *4*.

Stohl, E.A., and Seifert, H.S. (2001). The recX gene potentiates homologous recombination in Neisseria gonorrhoeae. Mol Microbiol *40*, 1301-1310.

Stohl, E.A., and Seifert, H.S. (2006). Neisseria gonorrhoeae DNA recombination and repair enzymes protect against oxidative damage caused by hydrogen peroxide. J Bacteriol *188*, 7645-7651.

Summers, C., Singh, N.R., White, J.F., Mackenzie, I.M., Johnston, A., Solanki, C., Balan, K.K., Peters, A.M., and Chilvers, E.R. (2014). Pulmonary retention of primed neutrophils: a novel protective host response, which is impaired in the acute respiratory distress syndrome. Thorax *69*, 623-629.

Sutherland, M., Schewe, T., and Nigam, S. (2000). Biological actions of the free acid of hepoxilin A3 on human neutrophils. Biochem Pharmacol *59*, 435-440.

Szabady, R.L., and McCormick, B.A. (2013). Control of neutrophil inflammation at mucosal surfaces by secreted epithelial products. Front Immunol *4*, 220.

Tager, A.M., and Luster, A.D. (2003). BLT1 and BLT2: the leukotriene B(4) receptors. Prostaglandins Leukot Essent Fatty Acids *69*, 123-134.

Tamang, D.L., Pirzai, W., Priebe, G.P., Traficante, D.C., Pier, G.B., Falck, J.R., Morisseau, C., Hammock, B.D., McCormick, B.A., Gronert, K.*, et al.* (2012). Hepoxilin A(3) facilitates neutrophilic breach of lipoxygenase-expressing airway epithelial barriers. J Immunol *189*, 4960-4969.

Tchoupa, A.K., Schuhmacher, T., and Hauck, C.R. (2014). Signaling by epithelial members of the CEACAM family - mucosal docking sites for pathogenic bacteria. Cell Commun Signal *12*, 27.

Thomas, C.P., Morgan, L.T., Maskrey, B.H., Murphy, R.C., Kuhn, H., Hazen, S.L., Goodall, A.H., Hamali, H.A., Collins, P.W., and O'Donnell, V.B. (2010). Phospholipid-esterified eicosanoids are generated in agonist-activated human platelets and enhance tissue factor-dependent thrombin generation. J Biol Chem *285*, 6891-6903.

Thomas, G.M., Carbo, C., Curtis, B.R., Martinod, K., Mazo, I.B., Schatzberg, D., Cifuni, S.M., Fuchs, T.A., von Andrian, U.H., Hartwig, J.H., *et al.* (2012). Extracellular DNA traps are associated with the pathogenesis of TRALI in humans and mice. Blood *119*, 6335-6343.

van der Ley, P., Heckels, J.E., Virji, M., Hoogerhout, P., and Poolman, J.T. (1991). Topology of outer membrane porins in pathogenic Neisseria spp. Infection and immunity *59*, 2963-2971.

van Putten, J.P. (1993). Phase variation of lipopolysaccharide directs interconversion of invasive and immuno-resistant phenotypes of Neisseria gonorrhoeae. EMBO J *12*, 4043-4051.

van Putten, J.P., and Paul, S.M. (1995). Binding of syndecan-like cell surface proteoglycan receptors is required for Neisseria gonorrhoeae entry into human mucosal cells. EMBO J *14*, 2144-2154.

Verjans, E.T., Zels, S., Luyten, W., Landuyt, B., and Schoofs, L. (2016). Molecular mechanisms of LL-37-induced receptor activation: An overview. Peptides *85*, 16-26.

Viola, A., and Luster, A.D. (2008). Chemokines and their receptors: drug targets in immunity and inflammation. Annu Rev Pharmacol Toxicol *48*, 171-197.

Virji, M. (2009). Pathogenic neisseriae: surface modulation, pathogenesis and infection control. Nat Rev Microbiol *7*, 274-286.

Waage, A., Halstensen, A., Shalaby, R., Brandtzaeg, P., Kierulf, P., and Espevik, T. (1989). Local production of tumor necrosis factor alpha, interleukin 1, and interleukin 6 in meningococcal meningitis. Relation to the inflammatory response. J Exp Med *170*, 1859-1867.

Wachter, J., and Hill, S. (2016). Positive Selection Pressure Drives Variation on the Surface-Exposed Variable Proteins of the Pathogenic Neisseria. PLoS One *11*, e0161348.

Walrand, S., Valeix, S., Rodriguez, C., Ligot, P., Chassagne, J., and Vasson, M.P. (2003). Flow cytometry study of polymorphonuclear neutrophil oxidative burst: a comparison of three fluorescent probes. Clin Chim Acta *331*, 103-110.

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

Wang, L.C., Yu, Q., Edwards, V., Lin, B., Qiu, J., Turner, J.R., Stein, D.C., and Song, W. (2017). Neisseria gonorrhoeae infects the human endocervix by activating non-muscle myosin II-mediated epithelial exfoliation. PLoS Pathog *13*, e1006269.

Weathington, N.M., van Houwelingen, A.H., Noerager, B.D., Jackson, P.L., Kraneveld, A.D., Galin, F.S., Folkerts, G., Nijkamp, F.P., and Blalock, J.E. (2006). A novel peptide CXCR ligand derived from extracellular matrix degradation during airway inflammation. Nat Med *12*, 317-323.

Wen, K.K., Giardina, P.C., Blake, M.S., Edwards, J., Apicella, M.A., and Rubenstein, P.A. (2000). Interaction of the gonococcal porin P.IB with G- and F-actin. Biochemistry *39*, 8638-8647.

Wenzel, S.E., Szefler, S.J., Leung, D.Y., Sloan, S.I., Rex, M.D., and Martin, R.J. (1997). Bronchoscopic evaluation of severe asthma. Persistent inflammation associated with high dose glucocorticoids. Am J Respir Crit Care Med *156*, 737-743.

Wi, T., Lahra, M.M., Ndowa, F., Bala, M., Dillon, J.R., Ramon-Pardo, P., Eremin, S.R., Bolan, G., and Unemo, M. (2017). Antimicrobial resistance in Neisseria gonorrhoeae: Global surveillance and a call for international collaborative action. PLoS Med *14*, e1002344.

Wiesner, P.J., and Thompson, S.E., 3rd (1980). Gonococcal diseases. Dis Mon *26*, 1-44.

Wilks, K.E., Dunn, K.L., Farrant, J.L., Reddin, K.M., Gorringe, A.R., Langford, P.R., and Kroll, J.S. (1998). Periplasmic superoxide dismutase in meningococcal pathogenicity. Infection and immunity *66*, 213-217.

Williams, I.R., and Parkos, C.A. (2007). Colonic neutrophils in inflammatory bowel disease: double-edged swords of the innate immune system with protective and destructive capacity. Gastroenterology *133*, 2049-2052.

Wira, C.R., Fahey, J.V., Sentman, C.L., Pioli, P.A., and Shen, L. (2005). Innate and adaptive immunity in female genital tract: cellular responses and interactions. Immunol Rev *206*, 306-335.

Wira, C.R., Ghosh, M., Smith, J.M., Shen, L., Connor, R.I., Sundstrom, P., Frechette, G.M., Hill, E.T., and Fahey, J.V. (2011). Epithelial cell secretions from the human female reproductive tract inhibit sexually transmitted pathogens and Candida albicans but not Lactobacillus. Mucosal Immunol *4*, 335-342.

Witkin, S.S., Linhares, I.M., and Giraldo, P. (2007). Bacterial flora of the female genital tract: function and immune regulation. Best Pract Res Clin Obstet Gynaecol *21*, 347-354.

Wixtrom, R.N., Silva, M.H., and Hammock, B.D. (1988). Affinity purification of cytosolic epoxide hydrolase using derivatized epoxy-activated Sepharose gels. Anal Biochem *169*, 71-80.

Woodhams, K.L., Chan, J.M., Lenz, J.D., Hackett, K.T., and Dillard, J.P. (2013). Peptidoglycan fragment release from Neisseria meningitidis. Infection and immunity *81*, 3490-3498.

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

Yarbrough, V.L., Winkle, S., and Herbst-Kralovetz, M.M. (2015). Antimicrobial peptides in the female reproductive tract: a critical component of the mucosal immune barrier with physiological and clinical implications. Hum Reprod Update *21*, 353-377.

Yokomizo, T., Izumi, T., Chang, K., Takuwa, Y., and Shimizu, T. (1997). A G-protein-coupled receptor for leukotriene B4 that mediates chemotaxis. Nature *387*, 620-624.

Yokomizo, T., Kato, K., Terawaki, K., Izumi, T., and Shimizu, T. (2000). A second leukotriene B(4) receptor, BLT2. A new therapeutic target in inflammation and immunological disorders. J Exp Med *192*, 421-432.

Yonker, L.M., Pazos, M.A., Lanter, B.B., Mou, H., Chu, K.K., Eaton, A.D., Bonventre, J.V., Tearney, G.J., Rajagopal, J., and Hurley, B.P. (2017). Neutrophil-Derived Cytosolic PLA2alpha Contributes to Bacterial-Induced Neutrophil Transepithelial Migration. J Immunol *199*, 2873-2884.

Yu, Z., Schneider, C., Boeglin, W.E., Marnett, L.J., and Brash, A.R. (2003). The lipoxygenase gene ALOXE3 implicated in skin differentiation encodes a hydroperoxide isomerase. Proc Natl Acad Sci U S A *100*, 9162-9167.

Zaman, K., Hanigan, M.H., Smith, A., Vaughan, J., Macdonald, T., Jones, D.R., Hunt, J.F., and Gaston, B. (2006). Endogenous S-nitrosoglutathione modifies 5-lipoxygenase expression in airway epithelial cells. Am J Respir Cell Mol Biol *34*, 387-393.

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

Zielke, R.A., Wierzbicki, I.H., Baarda, B.I., Gafken, P.R., Soge, O.O., Holmes, K.K., Jerse, A.E., Unemo, M., and Sikora, A.E. (2016). Proteomics-driven Antigen Discovery for Development of Vaccines Against Gonorrhea. Mol Cell Proteomics *15*, 2338-2355.

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

Zurawski, D.V., Mitsuhata, C., Mumy, K.L., McCormick, B.A., and Maurelli, A.T. (2006). OspF and OspC1 are Shigella flexneri type III secretion system effectors that are required for postinvasion aspects of virulence. Infection and immunity *74*, 5964-5976.