NEISSERIA INFECTION ACTIVATES CYTOPROTECTIVE PATHWAYS IN HUMAN EPITHELIAL CELLS

by

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CERTIFICATE OF APPROVAL

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Abstract

Over 300,000 cases of gonorrhea were recorded in 2009, making it the second most reported sexually transmitted infection in the U.S. However, the actual number of cases is likely much higher, as many gonorrhea infections are asymptomatic. The high incidence of asymptomatic infections indicates that *Neisseria gonorrhoeae*, the etiological cause of gonorrhea, can exist in the host undetected. The *Neisseria* genus also consists of several commensal species; bacteria that can infect their hosts without causing damage. Recent work has shown that *N. gonorrhoeae* evolved from the commensal *Neisseria*; this finding suggests that *N. gonorrhoeae* may have inherited traits from the commensals that help it establish asymptomatic infections. Indeed, *N. gonorrhoeae* possesses many strategies to minimize the host response to infection, including inhibiting apoptosis and downregulating the immune response. In this thesis, I demonstrate that *N. gonorrhoeae* represses production of pro-inflammatory cytokines by upregulating the host transcriptional regulator ATF3. Upregulation of ATF3 is enhanced by retraction of the Type IV pilus, a fimbriate structure that is expressed by the bacteria, and can enhance activation of host cytoprotective pathways by pulling on the host cell surface. I also show that the commensal *Neisseria elongata* upregulates ATF3 expression during infection. However, *N. elongata* induces lower levels of ATF3 expression when compared to *N. gonorrhoeae*. This result is consistent with the fact that commensal *Neisseria* induce lower levels of cytokine production than their pathogenic counterparts. Taken together, these data demonstrate a role for ATF3 in helping *Neisseria* species establish asymptomatic infections in their hosts.

Chapter 1:
Introduction

I. Research Significance

Neisseria gonorrhoeae, the etiological agent of the sexually transmitted disease (STD) gonorrhea, is a human-specific pathogen that infects over 62 million people worldwide each year [1]. While gonorrhea is treatable with antibiotics, a large number of infections are asymptomatic, and therefore go untreated. Asymptomatic infections are of particular concern for women; the majority of women infected with *N. gonorrhoeae* do not show obvious symptoms of disease [2]. Left untreated, gonorrhea can lead to salpingitis, ectopic pregnancy and infertility. The high incidence of asymptomatic infections indicates that *N. gonorrhoeae* can exist in its host undetected, and has developed strategies to modulate the immune response. Studying how *N. gonorrhoeae* manipulates the host response to infection will shed light on how this organism establishes and maintains itself asymptomatically in humans, and may lead to new therapies for treating and preventing gonorrhea.

II. *N. gonorrhoeae* Epidemiology

The *Neisseria* are a group of gram-negative β-proteobacteria that infect a variety of animals, including dogs, cats, rabbits, guinea pigs, iguanas, and monkeys [3, 4]. At least ten different species of *Neisseria* are known to infect humans; of these, eight are commensal bacteria, and only rarely cause disease [3, 5]. However, the remaining two human *Neisseria* species, *Neisseria meningitidis* and *N. gonorrhoeae*, make a significant impact on human health at the global level.

N. meningitidis is a major cause of bacterial meningitis. After initially colonizing the nasopharyngeal epithelium, it can cross the epithelial barrier and enter the bloodstream

to cause septicemia; from the bloodstream *N. meningitidis* can cross the blood-brain barrier to cause meningitis [6]. Meningococcal outbreaks occur annually in sub-Saharan Africa [7], where they are a leading cause of death among children 15 years and younger [8]. Despite its association with disease, *N. meningitidis* is not generally pathogenic. In non-epidemic regions, up to 35% of healthy individuals are asymptomatic carriers of *N. meningitidis* [9] and carriage can last for up to five months [10]. Increasingly, *N. meningitidis* is viewed as a commensal species that has the ability to cause opportunistic infections.

N. gonorrhoeae is generally considered pathogenic [5]. In 2009, the Centers for Disease Control and Prevention (CDC) recorded over 300,000 cases of gonorrhea in the United States, making it the second-most reported STD in this country. However, the actual number of new gonorrhea infections may be as high as 700,000 cases per year [11]. The large discrepancy between the numbers of reported and actual cases suggests that many individuals are unaware that they are infected with *N. gonorrhoeae*, and thus do not seek treatment. Indeed, asymptomatic gonorrhea infections occur in both men and women. For men, who clear gonococcal infections more readily than women, the rate of asymptomatic infection is approximately 5-15% [3, 12]. In women, unrecognized infections are much more common; the World Health Organization estimates that approximately 60% of all cervical gonorrhea infections are asymptomatic [2]. There is also evidence that *N. gonorrhoeae* can exist in a carriage state. A study of healthy individuals conducted in Baltimore in 2002 revealed that 5.3% of the population were asymptomatic carriers of *N. gonorrhoeae* [13]. These studies provide strong evidence that *N. gonorrhoeae*, similar to *N. meningitidis*, can behave like a commensal, by colonizing humans asymptomatically and only occasionally cause disease. At present,

the strategies that *N. gonorrhoeae* employs to induce asymptomatic infection are not well understood.

III. *N. gonorrhoeae* Pathogenesis: Clinical Aspects

N. gonorrhoeae is transmitted almost exclusively through sexual contact [14]. Primary infection occurs in the columnar or cuboidal mucosal epithelium of the urethra in men or the endocervix in women [15]. Clinically, gonorrhea comprises a broad range of symptoms, from uncomplicated local infections to systemic disease.

Infection of the Male Urethra

Uncomplicated acute urethritis is the most common manifestation of gonorrhea in men [15]. Infection of urethral epithelial cells is accompanied by a localized inflammatory response and the production of several pro-inflammatory cytokines [16]. These cytokines in turn attract neutrophils and other polymorphonuclear leukocytes (PMNs) to the site of infection. Infiltrating PMNs and shed epithelial cells constitute the purulent discharge that is characteristic of a fulminant gonococcal infection [15]. Symptoms, including urethral discharge and dysuria, usually appear two to five days after inoculation. Treatment of uncomplicated gonococcal urethritis with antibiotics is almost 100% effective in clearing the infection. The majority of male urethral infections can also spontaneously resolve themselves, usually within several weeks of the onset of symptoms. Gonorrhea-related complications in men are rare, but can include epididymitis, prostatitis, and seminal vesiculitis [17].

Infection of the Female Reproductive System

In women, the primary site of gonococcal infection is the endocervical canal. When symptoms are present they usually begin two to ten days post-inoculation and include vaginal discharge, dysuria, purulent cervical discharge and intermenstrual uterine bleeding [15]. Prompt treatment with antibiotics will effectively clear the infection in women. However, as previously noted, the majority of gonococcal infections in women are asymptomatic, and thus generally go untreated.

Due to its asymptomatic nature, gonorrhea infection in women is more likely to cause serious medical complications. Approximately 40% of untreated cervical infections will result in pelvic inflammatory disease (PID) [2], which occurs when the bacteria ascend into the upper reproductive tract to the endometrium, fallopian tubes, and ovaries [18]. Approximately 25% of women with PID will suffer from infertility, chronic pelvic pain, tubo-ovarian abscesses, and/or ectopic pregnancy [19]. In geographic areas with little access to health care, gonorrhea-related abscesses and ectopic pregnancy have a mortality rate of up to 30% [2].

Infection at Non-Urogenital Sites

Although *N. gonorrhoeae* is most frequently isolated from the urogenital tract, it will also readily infect other mucosal epithelial surfaces. Between 30 and 50% of women with gonococcal cervicitis will also have a concurrent rectal infection, which is usually the result of auto-inoculation [20]. Rectal infection is common in men who have sex with men (MSM), as a result of anal sexual intercourse [21]. MSM are also at a greater risk for pharyngeal gonococcal infection, as are women who engage in fellatio [22, 23].

Many rectal and pharyngeal gonorrhea infections are asymptomatic [21], and may represent reservoirs of *N. gonorrhoeae* within the population.

Prior to the practice of applying silver nitrate to the eyes of newborn infants, ocular gonorrhea was a significant factor in blindness [24]. Today, ocular gonorrhea occurs only occasionally in adults, and is usually a result of self-inoculation in individuals with a concurrent urogenital infection. Ocular gonorrhea is usually fulminant, with a purulent exudate and corneal ulcerations [17]. *N. gonorrhoeae* can cross the epithelial barrier to enter the bloodstream and cause septicemia, also known as Disseminating Gonococcal Infection (DGI). DGI is rare today; its clinical manifestations include acute arthritis, endocarditis, and meningitis [15].

Antibiotic Resistance in *N. gonorrhoeae*

Emerging antibiotic resistance is a major problem for the treatment of gonorrhea. *N. gonorrhoeae* has displayed widespread resistance to penicillins and tetracyclines since the early 1980s. In 2007, the CDC recommended discontinuing the use of fluoroquinolones for treatment of gonococcal infections, leaving cephalosporins as the only remaining option [25]. However, rates of cephalosporin resistance have steadily increased, and a strain of *N. gonorrhoeae* that is highly resistant to extended spectrum cephalosporin was recently reported [26, 27]. The development of new treatment options for gonorrhea is a major focus of research.

Commensals may serve as one source of antibiotic resistance for pathogenic *Neisseria*. Genome sequencing has revealed that human commensal *Neisseria* have the genetic capacity to donate DNA to and take up DNA from other *Neisseria* [28]. Recent work has

demonstrated that the commensal species *Neisseria elongata* can exchange DNA with *N. gonorrhoeae* in co-culture [29]. Furthermore, a *N. meningitidis* strain isolated from a mini-epidemic in 2007 appears to have acquired its ciprofloxacin resistance gene from another commensal species, *Neisseria lactamica*, that was harbored by a member of the local population [30].

IV. *N. gonorrhoeae* Pathogenesis: Experimental Models

Mammalian Models of Infection

Studies of *N. gonorrhoeae* are complicated by the bacteria's extreme adaptation to its human host. No animal model currently exists that recapitulates the characteristic symptoms observed in human gonorrhea infections. Experimental urethral inoculation of male volunteers has been used to study the early stages of infection in men [31]. These studies have characterized gonococcal virulence factors important for colonizing the human urethra [31-34] and identified cytokines produced in response to infection [16]. However, the usefulness of human studies is limited to the early stages of gonococcal infection, as subjects are given antibiotic treatment at the onset of symptoms. Furthermore, experimental infections can not be performed in women due to the risk of PID [31]. Thus, infection of the human endocervix has not been explored in vivo.

A recently developed mouse model shows promise for the study of *N. gonorrhoeae* vaginal and cervical infections. Infected mice recapitulate many important aspects of human infection, including production of pro-inflammatory cytokines and an influx of neutrophils to the site of infection; gram-negative diplococci can be observed in association with epithelial cells and neutrophils [35-37]. However, in contrast to human

cervical gonorrhea, the mice easily clear the infection, and asympotomatic colonization is not observed. The usefulness of the mouse model is limited by the host restrictions of *N. gonorrhoeae*. Mice do not express CD46 or CEACAMs, receptors that mediate gonococcal binding to human cells [38, 39]. Thus the interactions between *N. gonorrhoeae* and mouse epithelial cells may not be reflective of what occurs during natural gonococcal infections. Additionally, the *N. gonorrhoeae* receptors for transferrin and lactoferrin are highly specific for the human forms of these proteins [40]. The sources of iron for *N. gonorrhoeae* during mouse infection are unknown, but may include siderophores from other urogenital tract bacteria and hemin and ferritin from dying epithelial cells [41]. A transgenic human transferrin mouse was recently described, and may provide a more robust model of *Neisseria* infection [42].

Organ and Tissue Culture Models

Historically, human organ and tissue culture models have been used to study *N. gonorrhoeae* infection. Many of the molecular details of gonococcal-host cell interactions have been worked out in immortalized cell lines, as they are readily available and easy to manipulate. Organ and primary cell culture systems exist for both upper and lower portions of the female genital tract; these models have been used to confirm many earlier findings from immortalized cell lines [43]. The work presented in this thesis utilizes mucosal epithelial tissue culture models to study the early events of *N. gonorrhoeae* infection. Three different mucosal epithelial cell lines were used: End1 (endocervical), T84 (colorectal), and Detroit 562 (nasopharyngeal). Each of these lines is representative of a different site of infection in humans.

V. *N. gonorrhoeae* Pathogenesis: Cellular Aspects

Upregulation of Cytokine Production

Infection of mucosal epithelial tissues with *N. gonorrhoeae* causes a localized inflammatory response, the hallmark of which is an infiltration of neutrophils and other PMNs to the site of infection. Recruitment and differentiation of immature PMNs is mediated by chemokines and cytokines produced by infected cells, including those of the mucosal epithelium [44]. Cytokine induction by *N. gonorrhoeae* has been examined both in vivo and in vitro. A study in women with naturally occurring gonorrhea found elevated serum levels of IL-6 in infected patients [45]. However, none of the other cytokines measured were elevated in either the serum or in cervical secretions. This lack of cytokine production is consistent with the prevalence of asymptomatic infections in women, and the idea that *N. gonorrhoeae* can actively downregulate the immune response. In contrast, an experimental model of urethral infection showed that male volunteers infected with *N. gonorrhoeae* had elevated levels of the cytokines IL-1β, IL-6, IL-8, and TNFα in both the urine and plasma [16]. Interestingly, the authors observed that cytokine secretion in the urine occurred prior to the onset of symptoms such as purulent discharge. Thus, the mucosal epithelium may serve as an initial source of cytokines to recruit PMNs to the site of *N. gonorrhoeae* infection in men.

A number of studies have used organ and tissue culture models to examine cytokine levels during infection of epithelial surfaces. Human fallopian tube mucosa cultured with *N. gonorrhoeae* secrete significantly higher levels of TNFα and IL-1β when compared to uninfected controls [46, 47]. Both male urethral and endocervical epithelial cells secrete IL-6, IL-8, and TNF-α when cocultured with *N. gonorrhoeae*; endocervical tissue culture

cells have also been shown to produce GM-CSF and IL-1β [48-50]. It is interesting to note that both fallopian tube mucosa and endocervical cells secreted multiple cytokines upon infection with *N. gonorrhoeae*. These results contrast with the findings of Hedges, et al., who detected no cytokine induction in the cervical secretions of women with natural gonorrhea infections [45]. This discrepancy suggests that in vitro models of *N. gonorrhoeae* infection may not reflect what occurs during a case of human gonorrhea. However, it is important to note that due to the nature of the Hedges et al. study, it is not possible to ascertain the length of time that any of the subjects were infected prior to sample collection. Thus, because of the necessarily limited nature of gonorrhea studies in women, in vitro organ and tissue culture models can be valuable tools for examining cytokine induction by *N. gonorrhoeae*.

Infection of Mucosal Epithelial Surfaces

Mucosal epithelial cells are the primary site of infection for *N. gonorrhoeae*. Biopsies from infected patients show gonococci adjacent to and within epithelial cells [14]. Infection of human epithelial cells by *N. gonorrhoeae* has been extensively studied using organ explants and tissue culture. Based on these studies, a model for gonococcal infection of the mucosal epithelium has been proposed (Figure 1-1) [51].

In the first stage of infection, *N. gonorrhoeae* forms loose contacts with the epithelial host cell surface. Loose adherence is mediated by the Type IV pilus (Tfp, see below) [52-55], a fimbriate organelle found on the gonococcal outer membrane. Binding to the host cell occurs via interactions between Tfp and CD46, a eukaryotic integral membrane protein [39]. Tfp can also bind to the host cell in a CD46-independent manner [56], suggesting that multiple host cell receptors may function in pilus-mediated adherence.

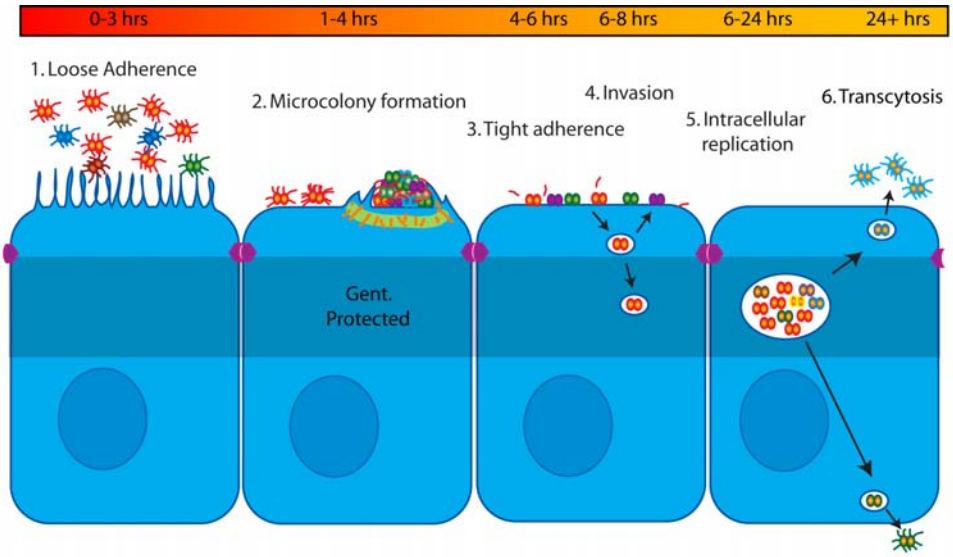

Figure 1-1. Timeline of *N. gonorrhoeae* infection in human epithelial cells. Tfp mediate loose adherance (1) to the mucosal epithelial cell, and microcolony and cortical plaque formation (2). Following microcolony dispersal, Opa proteins facilitate tight adhesion to (3) and invasion of (4) the cell. Gonococci replicate within the cell (5) in a gentimicin-protected compartment (Gent. Protected) before exiting the cell (6) either apically or basolaterally. See text for additional details. (Adapted from Wilbur [51].)

In the second stage of infection, individual gonococci begin to crawl together on the epithelial cell surface to form microcolonies [57]. Microcolony formation is readily observed one to four hours post-infection and is accompanied by the formation of cortical plaques within the host cell, directly beneath the microcolony [58]. Cortical plaques are structures in the eukaryotic plasma membrane that are dense with microvilli [59] and enriched for a number of host cell proteins thought or known to be important for the infection process. Components of cortical plaques include: actin, caveolae, CD44, CD46, EGFR, ezrin, ICAM-1, phosphoinositide-3 kinase (PI3K), and the Src kinase c-Yes [58, 60-63]. Cortical plaque proteins are important for many processes during infection, including attachment, invasion, and induction of host cell signaling cascades. Thus cortical plaques may function as coordination centers, bringing the structural and signaling components required for gonococcal infection into close proximity to the bacteria and each other.

At approximately six hours post-inoculation, *N. gonorrhoeae* starts to invade the epithelial cell. Invasion begins with the gonococci shedding their pili [64], a process which may prepare the bacteria for their later engulfment. Gonococci disperse from the microcolonies and form a monolayer on the cell surface. An intimate association is formed between the bacteria and the host cell, such that their membranes appear fused in electron micrographs [64]. Invasion is mediated by the Opas, a family of gonococcal outer membrane proteins. In epithelial cells, binding of host cell factors such as vitronectin and heparan sulfate proteoglycans by Opa50 [65-67] induces engulfment of the bacteria in a process that resembles macropinocytosis [68, 69]. Several cortical plaque proteins are also important for invasion. For example, inhibiting PI3K signaling reduces gonococcal invasion rates [63]. Recently, *N. gonorrhoeae* infection has been shown to induce EGFR kinase activity, and this activation is required for internalization of the bacteria [70].

Following invasion, gonococci can survive and replicate in a protected compartment within the host cell [54, 71, 72]. The exact nature of this compartment is unknown, but it is apparently inaccessible to the antibiotic gentamicin, as epithelial cells treated with gentamicin after inoculation yield viable bacteria [54, 72, 73]. Beginning at approximately 24 hours post-infection, newly replicated gonococci exit via the basolateral membrane [54, 74-76], where they may gain access to subepithelial tissues. Alternatively, bacteria may also exit the cell via the apical membrane [77], which provides them with the opportunity to re-infect the mucosal epithelium or be passed on to a new host. Notably, the entire infection process, from attachment and invasion to replication and exocytosis, does not disrupt tight junctions and causes minimal

cytotoxicity [54, 72]. These observations suggest that *N. gonorrhoeae* has developed strategies to co-exist with epithelial cells while causing minimal damage.

VI. Interactions Between Host Cells and Type IV Pili

Tfp Assembly

N. gonorrhoeae encodes multiple virulence factors that facilitate its interactions with epithelial cells. One important and well-characterized gonococcal virulence factor is Tfp, a fimbriate structure expressed on the outer membrane of the bacteria [78]. Tfp are expressed by many gram-positive and gram-negative bacteria [79, 80]. They have been shown to function in adhesion, DNA uptake, quorum sensing, and motility. The pilus fiber is made up of repeating subunits of pilin monomers, which are encoded by *pilE* [81]. Prepilin subunits are translated in the cytoplasm and translocated into the periplasmic space between the inner and outer membranes of the cell. In the periplasmic space, the PilD peptidase/transmethylase cleaves and methylates prepilin, resulting in mature, functional pilin monomers [82, 83]. Processed pilin monomers are then incorporated into the growing pilus fiber. Assembly of the pilus fiber requires the function of the ATPase PilF [83]. The mature fiber is extruded through and anchored by the outer membrane PilQ pore complex [84-86]. Retraction and disassembly of the pilus is mediated by a second ATPase, PilT [87, 88]. PilT is the strongest known biological motor, generating mechanical forces in the nanonewton range during Tfp retraction [89].

Tfp and Host Cell Signaling

Tfp mediate several important interactions between *N. gonorrhoeae* and the environment. Interestingly, many gonococcal-host cell interactions are enhanced by

pilus retraction. For example, Tfp retraction is required for microcolony and cortical plaque formation [58]. Pilus retraction is also important for stimulating proteolytic cleavage of CD46, a type I integral membrane protein that localizes to cortical plaques during infection. Gonococcal infection induces cleavage of CD46 by the presenilin/γ-secretase protease complex located in the host plasma membrane, and CD46 cleavage is greatly reduced in cells infected with *pilT* mutants compared to those infected with wild type *N. gonorrhoeae* [90].

Pilus retraction also enhances the induction of several host cell signaling pathways. Attachment to the host cell induces calcium fluxes in *N. gonorrhoeae*-infected cells [91], and the magnitude and duration of these fluxes is enhanced by Tfp retraction [Ayala 2005][92]. The PI3K and mitogen activated protein kinase (MAPK) pathways are both activated by gonococcal infection, and this activation is reduced in cells infected with *pilT* mutants [63, 93]. Activation of these signaling cascades may play an important role in protecting the host cell from the stress of infection. Both Tfp retraction and artificial pulling on the plasma can activate cytoprotective pathways that protect epithelial cells from apoptosis [93, 94]; this protection is mediated in part by signaling through the MAPK ERK [95].

Tfp and Host Cell Gene Expression

Bacterial infection has a profound effect on host cell gene expression. Microarray analysis has identified over 300 host genes whose expression is differentially regulated in response to *N. gonorrhoeae* infection [93]. A subset of these genes also have their expression enhanced by Tfp retraction. For example, *N. gonorrhoeae* infection *per se* upregulates expression of the *DUSP5* gene, and Tfp retraction further increases *DUSP5*

transcript levels [93]. Many retraction-enhanced genes encode proteins involved in cell stress and immune responses. Upregulation of many retraction-enhanced host genes can be blocked by inhibiting MAPK signaling [93], thus linking a retraction-enhanced signaling pathway to gene expression. Recent work has shown that activation of the NFκB transcription factor p65 is also elevated in epithelial cells infected with wild type *N. gonorrhoeae* compared to those infected with a *pilT* mutant [96]. As NFκB plays an important role in inducing cytokine production during infection, these data provides further evidence that pilus retraction is involved in regulating the innate immune response by epithelial cells. Intriguingly, one of the retraction-enhanced gene targets identified in the earlier microarray study was *ATF3*, which encodes a 23kDa protein involved in transcriptional regulation of the immune response.

VII. ATF3: Negative Regulator of Cytokine Expression

ATF3 belongs to the activating transcription factor/cyclic AMP response element-binding (ATF/CREB) family of eukaryotic transcription factors. ATF/CREB family members respond to a variety of environmental stimuli, but share the common function of preserving cellular homeostasis via transcriptional regulation [97]. All ATF/CREB transcription factors contain a basic-region leucine zipper domain (bZip) [98-100]. The basic region of this domain confers specific binding to the consensus cyclic AMP response element (CRE) DNA sequence, while the leucine zipper mediates homo- and heterodimerization with other bZip-containing proteins. Unlike most ATF/CREB family members, ATF3 is generally a repressor of transcription [101].

ATF3 was first isolated by screening clones from a cDNA library for their ability to bind the CRE consensus-binding site [98]. Subsequent studies have identified a variety of stimuli that upregulate ATF3, including: tissue wounding [102], endoplasmic reticulum stress [103-105], DNA damage [106-108], and both viral and bacterial infection [109-113]. ATF3 is also upregulated in a number of cancers, where it can either promote or inhibit oncogenesis, depending on the context [114-116]. Intriguingly, ATF3 expression can be induced by mechanical forces during ventilation-induced lung injury [117]. As ATF3 was also identified as a Tfp retraction-enhanced gene [93], these data indicate that mechanical force may play a role in inducing ATF3 expression during *N. gonorrhoeae* infection.

ATF3 Regulates Inflammatory Responses

Recent work has demonstrated that ATF3 is important for regulating the transcription of several pro-inflammatory cytokines. In a mouse model of allergic inflammation, ATF3 expression is upregulated in response to allergen stimulation [118]. ATF3 can then repress the transcription of allergen-induced cytokines such as IL-4, IL-5, and IL-13. ATF3 can also be induced by innate immune responses. In natural killer cells, ATF3 is expressed during murine cytomegalovirus (MCMV) infection [112]. In this model, ATF3 suppresses production of IFN-γ, an important cytokine for clearing viral infection. Thus, ATF3 expression actually promotes MCMV persistence by preventing the infection from being eliminated.

Several studies have demonstrated that ATF3 is induced by Toll-like receptor (TLR) signaling. TLRs are components of the innate immune system that “sense” infection by binding to molecular patterns that are commonly expressed by various microbes [119].

TLRs are widely expressed by a variety of cell types, including mucosal epithelial cells. Binding of different microbe-associated molecular patterns (MAMPs) to their respective TLRs activates pathways that are important for the innate immune response, including the production of pro-inflammatory cytokines and chemokines. However, excessive inflammation can be just as damaging to the host as an infection; TLR signaling must therefore be tightly controlled. Several lines of evidence suggest that ATF3 serves as a negative regulator of TLR inflammatory responses. ATF3 expression in bone marrow-derived macrophages is increased upon treatment with MAMP ligands for TLR2, TLR3, TLR4, TLR6, and TLR9 [120-122]. Furthermore, TLR activation induces elevated levels of the pro-inflammatory cytokines CCL4, IL-6, IL-12, and TNFα in *atf3*-deficient macrophages. Finally, *atf3*-deficient mice are more susceptible to lipopolysaccharide (LPS)-induced sepsis than wild type mice [120]. These findings demonstrate that, in addition to its roles in the adaptive immune response, ATF3 is an important negative regulator of TLR-mediated inflammation.

Mechanisms of ATF3 Regulation

ATF3 contains a leucine zipper domain that allows it to dimerize with other proteins [98]. Dimerization is important for ATF3 to function as a transcriptional regulator. ATF3 homodimers function as transcriptional repressors by binding to the CRE sequence in the promoter regions of target genes [103, 123]. ATF3 can also form heterodimers with other leucine zipper domain-containing proteins, including: ATF2, c-Jun, JunB, and JunD [98, 99, 104, 124, 125]. ATF3 heterodimers can either activate or repress transcription, depending on promoter context.

ATF3 can bind the promoter regions of many genes, and this binding is associated with transcriptional regulation of the targeted gene. However, in most cases the method of regulation has not been defined. The best characterized mechanism of ATF3-mediated transcriptional regulation is the repression of cytokine expression in macrophages and T cells. In LPS-treated macrophages, ATF3 recruits histone deacetylase 1 to the IL-6 promoter, and this recruitment is associated with increased deacetylase activity [120]. Deacetylation results in a less accessible chromatin structure, and thus represses gene expression [126]. Consistent with this idea, ovalbumin treatment induces higher levels of histone H4 acetylation at the IL-4, IL-5, and IL-13 promoters in *atf3*-deficient T cells compared to wild type T cells [118]. Thus, one method of ATF3-mediated repression is to promote a less accessible chromatin structure via histone deacetylation. It should be noted that ATF3 can also regulate cytokine expression indirectly. For example, in mouse macrophages, ATF3 inhibits expression of the transcription factor CEBPδ, which is a positive regulator of IL-6 transcription [127].

VIII. Relationship of *N. gonorrhoeae* to Commensal Species

At least eight species of commensal *Neisseria* are known to colonize healthy individuals [3]. Commensals are capable of causing opportunistic infections on rare occasions, especially in immunocompromised patients [3, 5, 128]. For example, both *N. elongata* and *Neisseria cinerea* have been isolated from patients with urogenital tract infections [129-131]. The fact that commensal and pathogenic *Neisseria* can infect the same mucosal surfaces suggests that they share common strategies for interacting with and persisting in humans.

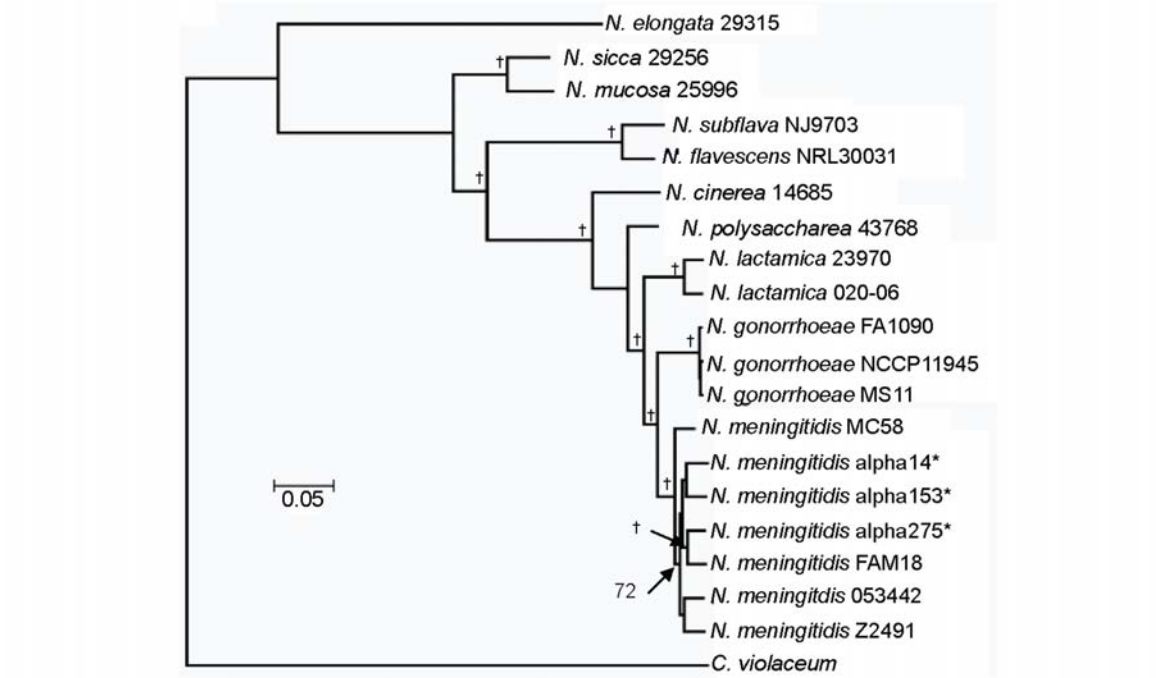

Figure 1-2. Phylogenetic relationship of the human *Neisseria* species. The rooted maximum likelihood tree is based on concatenating the DNA sequences of 636 core *Neisseria* genes that are also shared with the outgroup, *Chromobacterium violaceum*. A daggar denotes a bootstrap value of 100. Asterisks denote *N. meningitidis* carrier strains. (Adapted from Marri, et al., 2010.)

To gain insight into the genetic basis of commensal lifestyles, eight human commensal *Neisseria* species were sequenced and their genomes compared to the published genomes of 13 *N. meningitidis* and *N. gonorrhoeae* strains [28]. Using this information, a phylogenetic tree of the *Neisseria* genus was constructed (Figure 1-2). According to this tree, the two pathogenic *Neisseria* species evolved from the commensals. Commensal and pathogenic *Neisseria* have many genes in common, including a large number of virulence genes. Notable for this dissertation study, all *Neisseria* species encode a complete set of Tfp biogenesis genes.

Recently, *Neisseria elongata*, the most basal species on the *Neisseria* phylogenetic tree, was shown to produce Tfp that function in DNA transformation, attachment and

microcolony formation [29]. This study provides a mechanism—Tfp-mediated DNA transformation—for the extensive horizontal transfer of genetic information that has taken place among the *Neisseria* species. Given the many ways that Tfp modulates epithelial cell defenses during *N. gonorrhoeae* infection, the *N. elongata* findings suggest that commensal *Neisseria* may also use Tfp to alter host immune responses and create a favorable environment for colonization.

IX. Overview of Dissertation

Although considered a pathogen, *N. gonorrhoeae* frequently causes infections that are asymptomatic, particularly in women. The prevalence of asymptomatic infections suggests that *N. gonorrhoeae* has developed many strategies to minimize the host response to infection. When I began my dissertation research, *N. gonorrhoeae* had just been shown to activate cytoprotective pathways in epithelial cells. Activation of these pathways leads to increased expression of several host cell stress response genes, as well as protection from apoptosis. It was also shown that the cytoprotection is enhanced by Tfp retraction [93-95]. Inhibiting apoptosis may be one mechanism that helps *N. gonorrhoeae* to avoid causing overt clinical symptoms, and allows it to establish an asymptomatic infection. In this dissertation, I examine the upregulation of ATF3 during *N. gonorrhoeae* infection. As ATF3 is involved in downregulating the innate immune response, this pathway may also contribute to the ability of *N. gonorrhoeae* to exist in its host undetected.

Chapter 2 examines how *N. gonorrhoeae* upregulates expression of ATF3, and what role ATF3 plays during infection. ATF3 expression is increased in cells infected with *N.*

gonorrhoeae, and this expression is enhanced by Tfp retraction. ATF3 upregulation is blocked in infected cells that are pretreated with chemical inhibitors of the MAPKs. Thus, the MAPK signaling pathways are necessary for inducing ATF3 during *N. gonorrhoeae* infection. Knocking down endogenous levels of ATF3 results in higher levels of IL-6 transcript during infection. Thus, ATF3 appears to negatively regulate the expression of pro-inflammatory cytokines during *N. gonorrhoeae* infection.

During the course of my research, completion of the genomes for eight human commensal *Neisseria* species shed new light on the evolution of pathogenicity in *Neisseria* [28]. The commensal *Neisseria* are basal to the pathogens on the *Neisseria* phylogenetic tree. Thus, commensals are the oldest of the *Neisseria* species, and the pathogens evolved from the commensals. This finding suggests that the ability of *N. meningitidis* and *N. gonorrhoeae* to colonize the mucosa asymptomatically may have been inherited from their commensal ancestors. It also suggests that *N. meningitidis* and *N. gonorrhoeae* have acquired traits that allow them to be more pathogenic. To begin to understand what these traits are, we must compare how commensal and pathogenic *Neisseria* interact with mucosal epithelial surfaces, the primary site of infections for all *Neisseria* species. I chose to examine whether commensal *Neisseria* generate an immune response in epithelial cells, and to explore the nature of this response.

In Chapter 3, I examine the innate immune response in epithelial cells infected with the commensal species *N. elongata*. Preliminary results indicate that *N. elongata* infection induces cytokine production in epithelial cells. Consistent with an increase in cytokine expression, I observed an increase in the phosphorylation of the NFκB transcription factor p65 in *N. elongata* infected cells. Phosphorylation is associated with enhanced

transactivation activity by p65. ATF3 expression is also upregulated in response to *N. elongata* infection, although at lower levels than those seen during *N. gonorrhoeae* infection. These results suggest that *N. elongata* can induce the innate immune response in mucosal epithelial cells.

In the final chapter, I summarize the findings presented in this dissertation, and discuss their relevance to *Neisseria* pathogenesis. The host cell signaling pathways involved in ATF3 upregulation during *Neisseria* infection are discussed, as are additional potential targets for ATF3 transcriptional regulation. The significance of cytokine production during *Neisseria* infections is examined. Finally, I explore the potential of commensal *Neisseria* to activate cytoprotective pathways in epithelial cells. Together, these results lay the foundation for future experiments exploring the cytoprotective pathways that are activated during infection, and how these pathways contribute to asymptomatic infection by *Neisseria* species.

Chapter 2: Manuscript 1

Upregulation of ATF3 inhibits production of the pro-inflammatory cytokine IL-6 during *Neisseria gonorrhoeae* infection

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Summary

N. gonorrhoeae regulates the expression of many epithelial cell genes, activates cytoprotective pathways in the infected cell and protects it from apoptosis. We tested the hypothesis that *N. gonorrhoeae* modulates the innate immune response by inducing expression of ATF3, a transcription factor that negatively regulates the expression of many cytokine genes. We found that *N. gonorrhoeae* induces ATF3 expression in mucosal epithelial cells through activation of the mitogen activated protein kinases. Maximal ATF3 expression requires Type IV pilus retraction. Knocking down endogenous levels of ATF3 results in increased IL-6 transcript, suggesting that ATF3 is involved in suppressing cytokine expression during gonococcal infection.

Introduction

N. gonorrhoeae is a human-specific pathogen that infects over 62 million people annually [1]. While gonorrhea is treatable with antibiotics, a large number of infections are asymptomatic, and therefore go untreated. Asymptomatic infections are of particular concern for women; over 60% of women infected with *N. gonorrhoeae* do not show obvious symptoms of disease [2]. Left untreated, gonorrhea can lead to pelvic inflammatory disease, ectopic pregnancy and infertility [19]. The high incidence of asymptomatic infections indicates that *N. gonorrhoeae* has developed strategies to minimize the host response to infection.

The primary site of gonococcal infection in humans is the mucosal epithelium [3]. Although *N. gonorrhoeae* is most frequently isolated from the urogenital tract, it will also readily infect other mucosal epithelial surfaces, including rectal and pharyngeal epithelia

[15]. Attachment to the epithelial cell surface is mediated by Type IV pili (Tfp), which are fimbriate structures expressed on the outer surface of the bacteria [52-55]. Tfp mediate interactions between gonococci and their environment, including attachment to host tissues, DNA uptake, and bacterial twitching motility [132].

Many gonococcal-host cell interactions are enhanced by retraction of Tfp. Pilus retraction is mediated by PilT, an ATPase that can generate mechanical forces in the nanonewton range [87-89]. Tfp retraction is required for microcolony formation by *N. gonorrhoeae*, and the subsequent clustering of host cell proteins into cortical plaques beneath the microcolonies [58]. Proteolytic cleavage of the cortical plaque protein CD46 is enhanced in cells infected with wild type *N. gonorrhoeae* compared to those infected with a *pilT* mutant [90]. Pilus retraction also enhances activation of host cell signaling pathways, including the mitogen activated protein kinases (MAPKs) and phosphoinositide-3 kinase pathways [63, 93]. These signaling cascades may be important for protecting the host cell from the stress of infection. Pilus retraction activates cytoprotective pathways that protect epithelial cells from apoptosis [93, 94], and this protection is mediated in part by the MAP kinase ERK [95].

Tfp retraction can also influence host cell gene expression. Microarray analysis has identified over 300 host genes whose expression is differentially regulated in response to *N. gonorrhoeae* infection [93]. Tfp retraction enhances the level of expression of a subset of these genes. Many retraction-enhanced host genes encode proteins involved in cell stress and immune responses. One of these, activating transcription factor 3 (*ATF3),* encodes a 22 kD protein that is involved in transcriptional regulation.

ATF3 belongs to the activating transcription factor/cyclic AMP response element-binding (ATF/CREB) family of transcriptional regulators [97] ATF/CREB proteins, ATF3 contains a basic-region leucine zipper domain that allows it to form dimers and bind DNA [98, 99]. While most ATF/CREB proteins are involved in activating transcription, ATF3 generally acts as a transcriptional repressor.

Recently, ATF3 has been identified as an important regulator of inflammation. In a mouse model of allergic inflammation, ATF3 expression is upregulated in response to T cell activation [118]. ATF3 then represses the transcription of several allergen-induced cytokines. ATF3 is also expressed by natural killer cells during murine cytomegalovirus infection [112]. In this model, ATF3 suppresses production of IFN-γ, an important cytokine for clearing viral infection. ATF3 has also been shown to negatively regulate innate immune responses. Binding of different microbe-associated molecular patterns (MAMPs) to their respective Toll-like receptors (TLRs) leads to elevated levels of ATF3 in bone marrow-derived macrophages [120-122]. Additionally, TLR activation induces elevated levels of CCL4, IL-6, IL-12, and TNFα in *atf3*-deficient macrophages, indicating that ATF3 is required to repress transcription of these pro-inflammatory cytokines. Collectively, these studies demonstrate that ATF3 is an important regulator of cytokine expression by immune cells.

N. gonorrhoeae infection upregulates the expression of several cytokines by mucosal epithelial tissues [16, 45, 46, 48, 49]. Of these, IL-6 and TNFα are known to be regulated by ATF3 [120, 122]. However, the role of ATF3 in regulating the immune response of *N. gonorrhoeae*-infected epithelial cells has not been explored. We hypothesize that ATF3

expression in mucosal epithelial cells is induced by *N. gonorrhoeae* infection and that ATF3 expression helps dampen the host response to infection by inhibiting transcription of pro-inflammatory cytokines. We demonstrate that *N. gonorrhoeae* infection upregulates ATF3 expression. We also show that ATF3 upregulation is enhanced by Tfp retraction, and requires signaling via MAPK pathways. Finally, we present evidence that ATF3 regulates expression of the pro-inflammatory cytokine IL-6 during *N. gonorrhoeae* infection.

Results

ATF3 is upregulated during *N. gonorrhoeae* infection

Our previous microarray analysis indicated that the expression of a large number of host cell genes may be altered during *N. gonorrhoeae* infection [93]. One target identified by that study was the immunoregulatory factor ATF3. To verify that ATF3 expression is induced by infection, T84 human colorectal epithelial cells were infected with *N. gonorrhoeae* strain MS11, and relative ATF3 transcript levels assessed by RT-PCR. At both three and six hours post-infection, ATF3 transcript levels were significantly higher in MS11-infected cells than in uninfected cells (Figure 2-1A, $p<0.001$). To show that ATF3 protein levels are also upregulated during infection, western blots of T84 whole cell lysates were probed with an antibody specific for ATF3. ATF3 protein levels were significantly higher in MS11-infected samples than uninfected samples as early as three hours post-infection and remain elevated throughout the course of infection (Figure 2-1B and 2-1C). The two ATF3 isoforms migrate as a doublet [116] and both are upregulated by infection. ATF3 upregulation was also observed in MS11-infected endocervical (End1), nasopharyngeal (Detroit 562), and bronchial (16HBE14o-) epithelial cells, and in

T84 cells in response to infection with *N. gonorrhoeae* strain FA1090 and the *Neisseria meningitidis* strain 8013.

Tfp retraction enhances the level of expression of many of the stress response genes that are upregulated by *N. gonorrhoeae* infection [93, 133]. To determine whether pilus retraction plays a role in ATF3 expression, we compared ATF3 levels in cells infected with wild type (wt) MS11 to those of cells infected with a retraction-deficient mutant MS11Δ*pilT* strain [133]. ATF3 transcript levels are elevated in cells infected with the Δ*pilT* strain ($p<0.001$). However, while Δ*pilT* does upregulate ATF3, the levels are significantly lower than those seen with MS11 (Figure 2-1A). Similarly, infection with Δ*pilT* results in increased expression of ATF3 protein when compared to uninfected cells, but in lower amounts than wild type infection (Figure 2-1B and 2-1C). An MS11Δ*pilE* strain induced ATF3 transcript and protein levels similar to those seen with the Δ*pilT* mutant (data not shown). Thus, Tfp expression is not essential for ATF3 expression. However, Tfp expression and retraction are important for inducing maximum levels of ATF3. Collectively, these data demonstrate that ATF3 is upregulated in response to *Neisseria* infection in a variety of epithelial cell types, and that ATF3 expression is enhanced by Tfp expression and retraction.

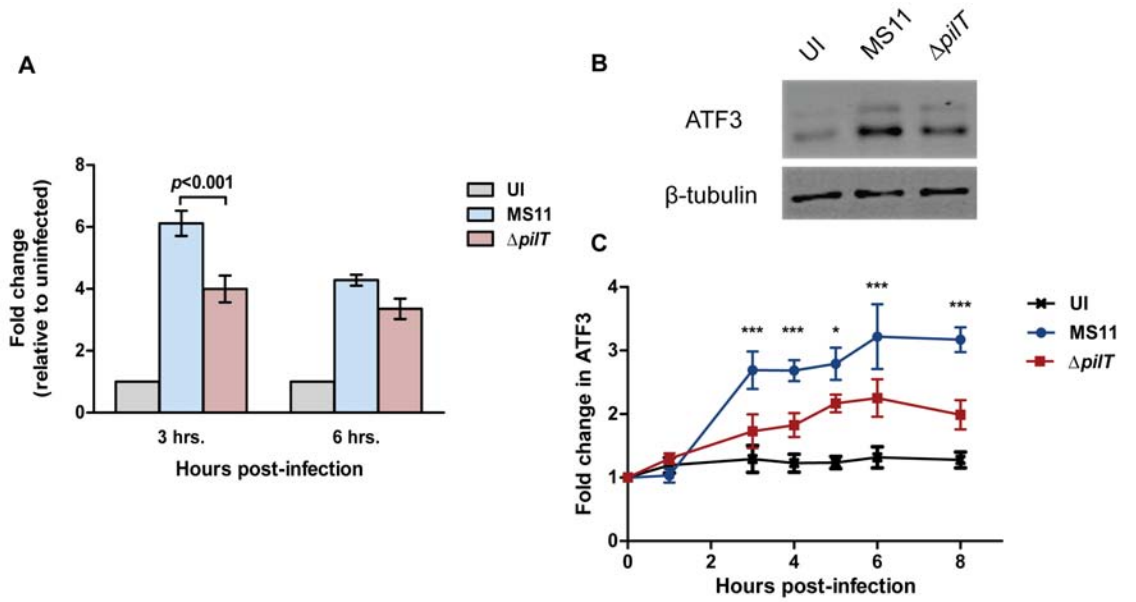

Figure 2-1. ATF3 is upregulated in response to *N. gonorrhoeae* infection and its expression is enhanced by Tfp retraction. T84 cells were infected with wild type strain MS11 or Δ*pilT*. (A) ATF3 transcript levels at three and six hours post-infection were assessed by RT-PCR. Values are expressed as mean relative transcript levels, normalized to GAPDH, with the uninfected (UI) sample for each time point set at one (±SEM, n=4). (B) Representative western blot of ATF3 induction in T84 cells at three hours post-infection. Tubulin was used as a loading control. (C) Time course of ATF3 expression. Samples were collected at the indicated time points, subjected to western blot, and relative ATF3 levels assessed by densitometry. Values represent mean normalized ATF3 protein levels, relative to the zero hour time point, which is set at one (±SEM, n=6). A single asterisk denotes a *p* value <0.05, triple asterisks signify a *p* value of <0.001.

ATF3 expression is induced by a *N. gonorrhoeae* outer membrane protein

Purified lipopolysaccharide (LPS) is sufficient to induce ATF3 expression in macrophages [120-122], suggesting that live bacteria are not essential for ATF3 expression. We therefore examined whether live bacteria are necessary to upregulate ATF3 in epithelial cells. T84 cells were treated with increasing amounts of crude MS11 outer membrane preparations (OMP), and ATF3 levels assessed by western blotting. As little as 10 μg/ml of OMP was sufficient to induce significant ATF3 upregulation in epithelial cells (Figure 2-2A and 2-2B).

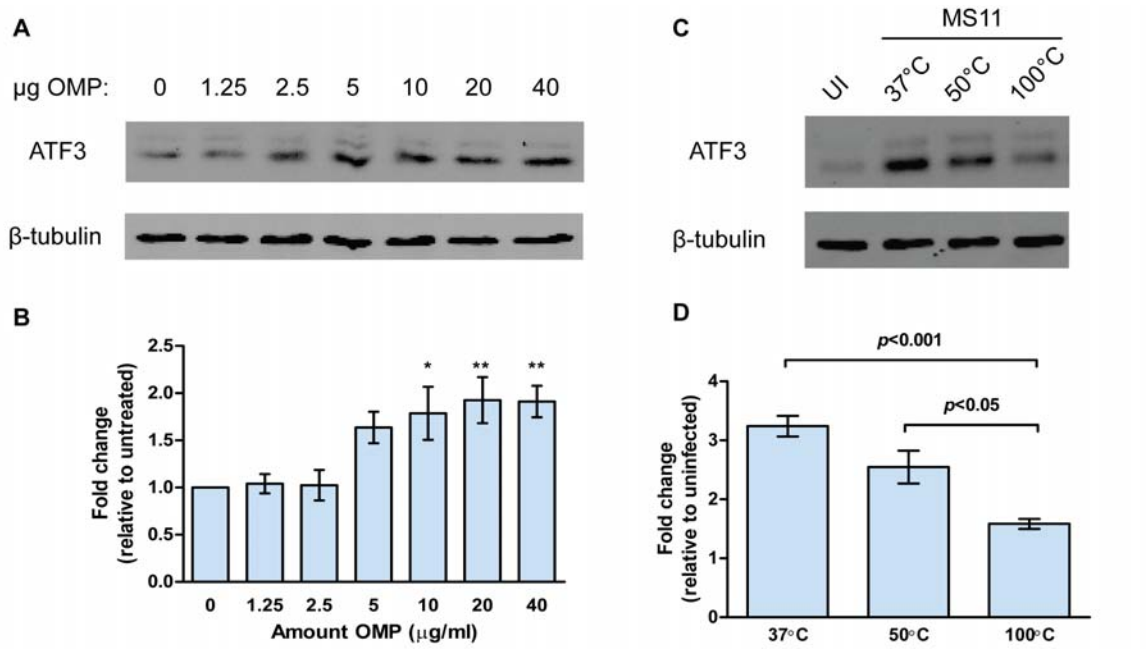

Figure 2-2. *N. gonorrhoeae* outer membrane protein induces ATF3 upregulation. (A) T84 cells were treated with increasing amounts of crude outer membrane preparations (OMP) isolated from MS11 and assessed for ATF3 protein levels at four hours post-treatment. Tubulin was used as a loading control. (B) Densitometry values from (A) are expressed as mean normalized ATF3 protein levels; cells treated with 0 μg OMP are set at one (±SEM, n=4). A single asterisk indicates a *p* value of <0.05, double asterisks denote a *p* value of <0.01. (C) ATF3-inducing factor is heat sensitive. MS11 was incubated at 37°C, 50°C, or 100°C for 30 minutes prior to infection of T84 cells. Samples were collected at three hours post-infection and protein levels assessed by western blot. (D) Densitometry values from (C) represent mean normalized ATF3 protein levels (±SEM, n=4).

ATF3 expression can be activated by multiple TLRs, including TLR4 in response to LPS treatment and TLR2 in response to zymosan [120, 122]. As the cell lines that we used express little to no TLR4 [134-136], LPS is unlikely to be important for ATF3 upregulation during gonococcal infection. However, at least two neisserial outer membrane proteins, PorB and Lip, have been shown to induce TLR2-mediated signaling [137, 138]. To determine whether gonococcal proteins are important for upregulating ATF3 expression, we infected epithelial cells with wt MS11 that had been pre-incubated for 30 minutes at 37°C, 50°C (a temperature sufficient to kill bacteria, but maintain the structure of most proteins), or 100°C (which will denature proteins but not glycans such as LPS). ATF3 protein levels are still elevated in cells infected with *N. gonorrhoeae* pre-incubated at

50°C (Figure 2-2C and 2-2D); this is consistent with the earlier finding that live bacteria are not required to upregulate ATF3. In contrast, cells infected with 100°C-treated bacteria expressed significantly lower levels of ATF3 when compared to other temperature treatments. As heat treatment at 100°C is sufficient to denature proteins, this suggests that *N. gonorrhoeae* protein may be necessary to induce ATF3 upregulation. This interpretation is supported by experiments with heat-killed *N. gonorrhoeae* that were treated with proteinase K (PK) to degrade surface proteins. T84 cells infected with the PK-treated bacteria expressed reduced levels of ATF3 when compared to cells infected with untreated Ng (Figure 2-S1). Taken together, these results indicate that *N. gonorrhoeae* outer membrane proteins are responsible for inducing a significant portion of the ATF3 that is expressed during infection.

MAPK signaling is required for *N. gonorrhoeae*-induced ATF3 expression

MAPKs are a family of serine/threonine kinases that are activated by environmental stimuli [139]. The three major MAPK groups, ERK, p38, and JNK, help maintain cellular homeostatsis by upregulating expression of immediate early stress response genes, including ATF3 [140-142]. MAPKs respond to a variety of stimuli, including growth-promoting mitogens, environmental stresses like heat shock and radiation, and pro-inflammatory stimuli such as cytokines and microbial products [143]. Both *N. gonorrhoeae and N. meningitidis* activate MAPK signaling [144, 145]; in the case of *N. gonorrhoeae* this activation is enhanced by Tfp retraction [93]. Several neisserial outer membrane proteins have been shown to activate MAPKs [145-147]. We therefore hypothesized that the MAPKs may act upstream of *N. gonorrhoeae*-induced ATF3 expression. We therefore determined whether inhibitors of ERK, p38, and JNK can

block ATF3 expression during infection. T84 cells were pre-incubated with inhibitors U0126 (ERKi), 203580 (p38i) and SP600125 (JNKi) individually or together (MAPKi), then infected with MS11. We have previously shown that these inhibitors block *N. gonorrhoeae*-induced MAPK signaling in T84 cells [93]. Each inhibitor blocked infection-induced ATF3 upregulation to a small but significant extent (Figure 2-3A and 2-3B). When pooled, the three inhibitors completely blocked ATF3 upregulation. These experiments indicate that MAPK signaling is required to induce ATF3 expression.

Protein kinase R (PKR) is another stress response kinase that has been shown to regulate ATF3 expression [148]. We found that treating with a chemical PKR inhibitor blocked infection-induced ATF3 expression, while PKR siRNA had no effect. These results suggest that the activity of the PKR inhibitor on ATF3 expression is likely due to an off-target effect (Figure 2-S2).

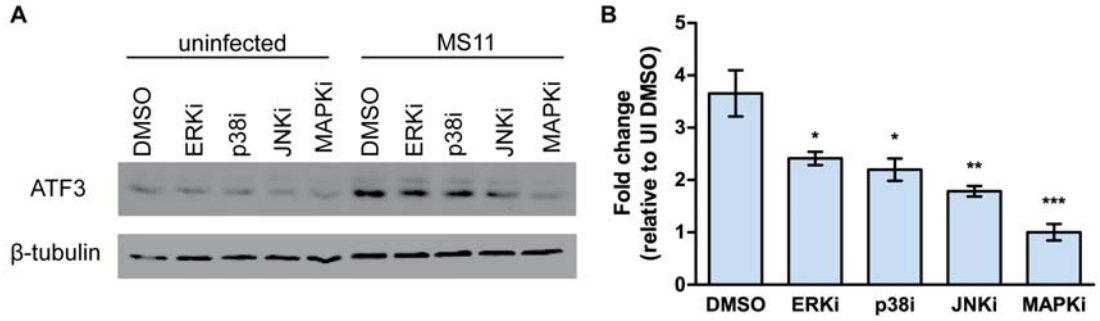

Figure 2-3. MAP kinase signaling is required for ATF3 induction. T84 cells were incubated with the chemical inhibitors U0126, 203580, or SP600125 individually (ERKi, p38i, or JNKi, respectively) or in combination (MAPKi) at a final concentration of 10 μM for one hour prior to infection with MS11. (A) Representative western blot of ATF3 protein levels in the presence and absence of MAP kinase inhibitors. Total β-tubulin was used as a loading control. (B) Densitometry values from MS11-infected samples in (A) are expressed as mean, normalized ATF3 protein levels (±SEM, n=4). A single asterisk represents a *p* value of <0.05, double asterisks indicate a *p* value of <0.01, and triple asterisks denote a *p* value of <0.001.

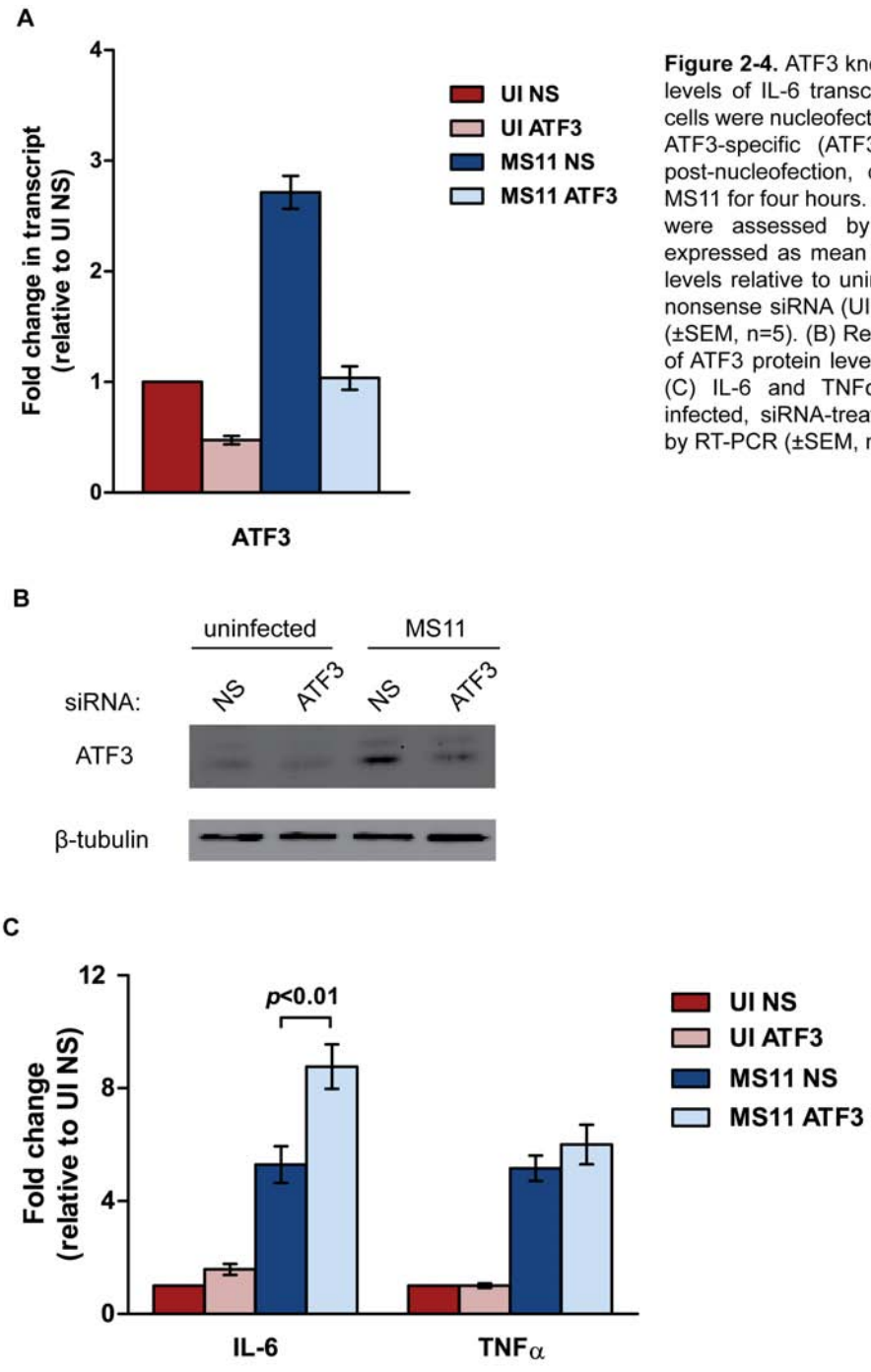

Figure 2-4. ATF3 knockdown results in higher levels of IL-6 transcript during infection. T84 cells were nucleofected with nonsense (NS) or ATF3-specific (ATF3) siRNA. At 72 hours post-nucleofection, cells were infected with MS11 for four hours. (A) ATF3 transcript levels were assessed by RT-PCR. Results are expressed as mean fold change in transcript levels relative to uninfected cells treated with nonsense siRNA (UI NS), which is set at one (±SEM, n=5). (B) Representative western blot of ATF3 protein levels in siRNA-treated cells. (C) IL-6 and TNFα transcript levels from infected, siRNA-treated cells were assessed by RT-PCR (±SEM, n=5).

ATF3 negatively regulates IL-6 expression during *N. gonorrhoeae* infection

Given the role of ATF3 in negatively regulating cytokine expression by immune cells, we hypothesized that ATF3 may function similarly in *N. gonorrhoeae*-infected mucosal epithelial cells. We knocked down ATF3 expression in T84 cells using siRNA, then assessed the levels of TNFα and IL-6, two cytokines known to be induced by *N. gonorrhoeae* and regulated by ATF3. T84 cells were nucleofected with an ATF3-specific siRNA or a nonsense (NS) siRNA and infected with MS11 at 72 hours post-nucleofection. ATF3 expression was successfully knocked down by ATF3 siRNA, but not NS siRNA (Figure 2-4A and 2-4B). Infection of ATF3 siRNA-treated cells resulted in higher levels of IL-6 than NS siRNA-treated cells (Figure 2-4C). These results strongly suggest that ATF3 is involved in suppressing infection-induced expression of IL-6. Knocking down ATF3 with siRNA did not alter the ability of *N. gonorrhoeae* to induce TNFα.

Tfp retraction enhances IL-6 expression

Retraction-deficient *N. gonorrhoeae* is less able to upregulate ATF3 (Figure 2-1). This suggests that such a mutant is also less able to induce IL-6 expression. We therefore compared IL-6 transcript levels in T84 cells infected with MS11 and MS11Δ*pilT*. At three hours post-infection, both strains induced significant upregulation of IL-6 compared to uninfected cells ($p<0.001$). However, wt-infected cells had significantly more IL-6 transcript than Δ*pilT*-infected cells (Figure 2-5). By six hours post-infection, both wt and Δ*pilT*-infected cells had reduced levels of IL-6 transcript. However, they are significantly higher than the levels seen in uninfected cells, and wt-infected cells still had slightly higher levels of IL-6 mRNA than Δ*pilT*-infected cells. These results indicate that IL-6

expression is induced by *N. gonorrhoeae* expressing Tfp, and enhanced by Tfp retraction.

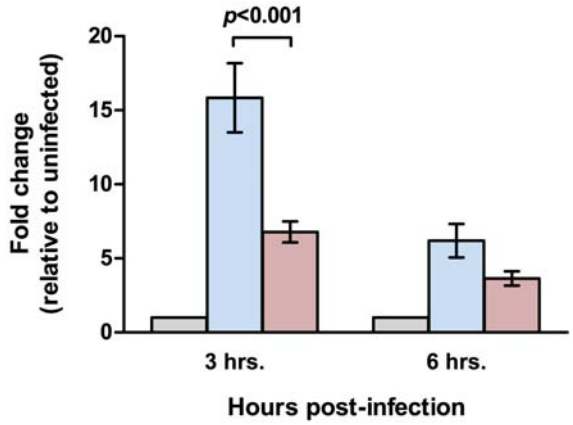

Figure 2-5. Tfp retraction enhances IL-6 transcription. T84 cells were infected with MS11 or Δ*pilT* and assessed for IL-6 transcript levels at three and six hours post-infection. Values are expressed as mean relative transcript levels, normalized to GAPDH, with the uninfected sample for each time point set at one (±SEM, n=4).

Discussion

We have provided evidence that *N. gonorrhoeae* upregulates expression of ATF3, a member of the ATF/CREB family of transcriptional regulators, through the three MAPK cascades (Figures 2-1 through 2-3). Further, we identified a role for ATF3 in *N. gonorrhoeae* infection; specifically, ATF3 negatively regulates expression of the pro-inflammatory cytokine IL-6 (Figure 2-4). The role of ATF3 in regulating the innate response to infection is well characterized in professional immune cells [101] but has not been studied in mucosal epithelial cells, which represent the first line of defense against microbes. To our knowledge, our study is the first to demonstrate a role for ATF3 in regulating epithelial cell responses to infection.

Tfp retraction is necessary to induce maximal expression of ATF3 during *N. gonorrhoeae* infection (Figure 2-1). ATF3 expression can also be induced by crude

OMPs (Figure 2-2) and by infection with the relatively non-adherent MS11Δ*pilE* mutant (data not shown) [54]. These results suggest that *N. gonorrhoeae* induces ATF3 through Tfp-dependent and -independent pathways. In this model, bacterial components other than Tfp initially induce ATF3 expression at low levels, and Tfp retraction subsequently enhances this expression. Thus ATF3 belongs to the set of retraction-enhanced genes that promotes a cytoprotective state in the epithelial cell.

MAPK signaling is required for *N. gonorrhoeae* upregulation of ATF3 (Figure 2-3). Previous work has shown *Neisseria* infection activates MAPK signaling [93, 144, 145]. Importantly, activation of all three MAPK pathways is enhanced by Tfp retraction, and by mechanical forces generated artificially on the epithelial cell membrane [93]. Recently, mechanical force was shown to induce ATF3 expression during ventilation-induced lung injury [117]. Enhanced ATF3 expression in *N. gonorrhoeae*-infected cells may therefore result from increased MAPK activation by mechanical forces exerted on the epithelial cell by Tfp retraction.

Wt bacteria induce significantly higher levels of IL-6 than the *pilT* mutant at early time points (Figure 2-5). Previous reports have also shown that Tfp retraction exerts its effect on cytokine expression early in infection [93, 96]. These observations appear to be inconsistent with the role of Tfp in promoting cytoprotection. However, the induction of specific cytokines may actually be beneficial for *N. gonorrhoeae*. For example, IL-6 secretion can inhibit apoptosis of cervical cancer cells by upregulating expression of the pro-survival protein MCL-1 [149]. Given that *pilT* mutants induce greater levels of apoptosis [93-95], it is tempting to speculate that the cytoprotective effects of Tfp retraction are mediated in part through a transient upregulation of IL-6 expression. By six

hours post-infection, IL-6 transcript levels have dropped significantly (Figure 2-5), which is consistent with the increased level of ATF3 expression at this time point (Figure 1). Thus, early expression of IL-6 may help *N. gonorrhoeae* establish infection by preventing epithelial cell death, while ATF3 expression at later time points prevents excessive inflammation that would be damaging to both host and microbe. It will be interesting to test whether IL-6 expression helps to protect infected cells from apoptosis in future experiments.

Mucosal epithelial cells are the primary site of infection for many microbes. The ability of *N. gonorrhoeae* to induce a cytoprotective state in these cells is likely to be a key step in establishing asymptomatic infections. We have demonstrated that *N. gonorrhoeae* upregulates expression of the transcriptional regulator ATF3 during infection of epithelial cells; in turn, ATF3 inhibits production of the pro-inflammatory cytokine IL-6. These studies provide the basis for future work examining how repression of the host immune response can promote asymptomatic *N. gonorrhoeae* infections.

Materials and Methods

Reagents

The following antibodies were used in this study: anti-ATF3 (C-19, Santa Cruz Biotechnology) and anti-β-tubulin (E7, University of Iowa Developmental Studies Hybridoma Bank). Assays on Demand TaqMan probes for GAPDH (Hs99999905_m1), ATF3 (Hs00231069_m1), IL-6 (Hs00174131_m1), and TNFα (Hs00174128_m1) were obtained from Applied Biosystems. ATF3-specific and non-specific control siRNAs were

purchased from Dharmacon RNAi Technologies. The MAP kinase inhibitors U0126 (ERK), 203580 (p38) and SP600125 (JNK) were purchased from Calbiochem and used as described previously [93] at a final concentration of 10 μM.

Tissue culture

T84 colorectal epithelial cells (ATCC #CCL-248) were maintained at 37°C with 5% CO_2 in Dulbecco's modified Eagle medium containing Ham's F12 nutrient mixture and 5% heat-inactivated FBS (DMEM/F12/5% FBS). Cells were passaged every four to five days and only used up to a passage number of 12. Unless otherwise stated, cells were seeded into 6-well tissue culture plates and used at approximately 80-90% confluency.

Bacterial strains and infections

The *N. gonorrhoeae* strains MS11 and MS11Δ*pilT* [133] were used in this study. Strains were grown on gonoccocal medium base (GCB) agar (Difco) with Kellogg's supplements I and II at 37°C with 5% CO_2. Piliation and Opa phenotypes were determined by observing colony morphology using light microscopy. Only piliated, Opa non-expressing bacteria were used. For infections, 18-hour-old bacteria were swabbed from GCB agar and resuspended in liquid GCB medium. Epithelial cells were infected with bacteria at a multiplicity of infection (MOI) of 50, or mock infected with GCB liquid medium.

Crude outer membrane preparations

N. gonorrhoeae outer membrane preparations (OMPs) were prepared as previously described [150]. Briefly, 18-hour-old bacteria were swabbed from GCB agar and resuspended in Hank's Balanced Salt Solution (HBSS). Samples were vigorously

vortexed for two minutes followed by centrifugation at 16,100 × *g* for five minutes at 4°C to remove whole bacteria. Clarified supernatants were used immediately or stored at -80°C. Total protein was quantified by BCA assay (Pierce).

Heat-treated bacteria

Bacteria were resuspended in GCB liquid medium and incubated at 37°C, 50°C, or 100°C for 30 minutes. Samples were then briefly vortexed and epithelial cells infected with bacteria at or equivalent to an MOI of 50. Aliquots of heat-treated bacteria were also plated onto supplemented GCB agar and incubated at 37°C with 5% CO_2 for 48 hours to ensure complete killing.

MAP kinase inhibition

T84 cells were incubated in serum-free DMEM/F-12 for 18 hours prior to infection. On the day of infection, the media was aspirated off and replaced with fresh serum-free media containing either the Erk, p38, or Jnk chemical inhibitors individually, or with media containing all three inhibitors at once. Control samples were treated with serum-free media containing the vehicle (DMSO) alone. Cells were incubated in the inhibitors for one hour before being infected with MS11 at an MOI of 50.

RNA isolation

T84 cells were lysed in buffer RLT (Qiagen) and scraped into Qiashredder columns to homogenize the samples. Total RNA was purified using the Qiagen RNeasy mini-kit (Qiagen). Purified RNA samples were eluted in RNA Storage Solution (Ambion) and stored at -80°C.

Real-time PCR

For each sample, one microgram of total RNA was transcribed into cDNA using the iScript Select cDNA Synthesis kit (Biorad) according to the manufacturer's instructions. Semi-quantitative real-time PCR was performed on an ABI Prism 7300 using TaqMan Universal master mix (Applied Biosystems) pre-designed TaqMan probes, as per the manufacturer's protocols. Reactions were performed in a 20 μl volume in triplicate. Transcript levels were normalized to the housekeeping gene GAPDH, and relative expression values determined using the comparative Ct method.

Preparation of protein lysates

Cells were washed two times with ice cold PBS and then lysed in ice cold RIPA buffer (10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1% SDS, 1% deoxycholate, 1% Triton X-100) supplemented with 1X Complete, EDTA-free protease inhibitor cocktail (Roche) and 1X PhosSTOP phosphatase inhibitor cocktail (Roche). Samples were incubated on ice for 20 minutes before being scraped into microfuge tubes. Nuclei and insoluble debris was removed by centrifugation at 16,100 × *g* for 20 minutes at 4°C. Cleared lysates were stored at -80°C until further processing.

Immunoblotting

Protein samples were resolved by SDS-PAGE and transferred onto a 0.45 μm nitrocellulose membrane (GE Osmonics). Blots were blocked for one hour in Tris-buffered saline (TBS) containing 0.1% Tween and 5% nonfat dry powdered milk. Primary antibodies were diluted in either 5% milk/TBST (β-tubulin) or 5% BSA/TBST (ATF3) and incubated overnight at 4°C. The blots were imaged on the LICOR Odyssey Infrared

Imaging System, and band intensities were measured by densitometry using Image J v1.36b.

RNA interference

T84 cells were nucleofected with siRNA using the Amaxa Nucleofector II as previously described [95]. For each nucleofection, approximately 1×10^6 cells were aliquotted into a microfuge tube and centrifuged at 180 × *g* for two minutes. Residual medium was removed by aspiration and the cell pellet resuspended in 100 μl of Nucleofector Solution R (Lonza). For each sample, 2.56 nmol of siRNA was added and mixed by gentle flicking. The suspension was transferred to an electroporation cuvette and nucleofected on setting T-016. Following nucleofection, 400 μl of pre-warmed RPMI/10% NCS was added; the mixture was then transferred to a clean microfuge tube and incubated in a heat block at 37°C for 5-10 minutes. Cells were seeded into one well of a 12-well plate containing an additional 1.5 ml of pre-warmed DMEM/F12/5% FBS. At 72 hours post-nucleofection, the cells were infected with MS11 at an MOI of 50. After four hours, RNA was isolated and relative transcript levels were assessed by RT-PCR as described above. After binding nucleic acid to the column, the remaining flow through was saved. One part of flow through was combined with four parts ice cold acetone, briefly vortexed and incubated overnight at -20°C. The precipitate was then centrifuged at 16,100 × *g* for 5 minutes at 4°C. The pellet was washed once with ice cold 100% ethanol, dried briefly and resuspended in 100 μl 1X SDS-PAGE sample buffer supplemented with protease and phosphatase inhibitors. ATF3 protein levels were then assessed by western blotting.

Statistics

Statistical analyses were performed using Prism 5 (GraphPad Software). Significant differences were determined by one-way or two-way ANOVA, followed by Bonferroni's post hoc comparison test. Outliers were eliminated using Grubbs' test.

Acknowledgements

We would like to thank D. Vercelli for use of the AMAXA nucleofector for our RNA interference experiments. We are grateful to D.R. Hernández for help with statistical analysis and members of the So lab for their thoughtful suggestions about this manuscript. This work was supported in part by NIH grant RO1 AI068033 to M. So.

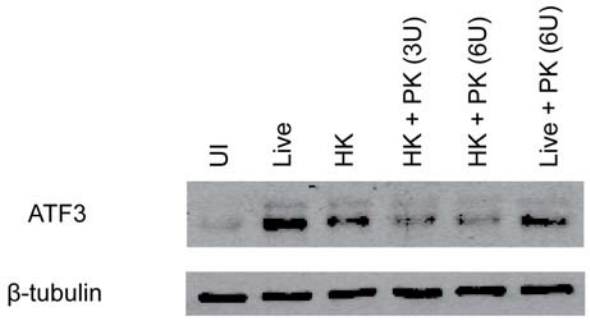

Figure 2-S1. Gonococcal protein is important for ATF3 induction. MS11 was incubated at 37°C (Live) or 50°C (HK) for 30 minutes and then treated with 3 or 6 units of proteinase K (PK) for one hour prior to infection. Tubulin serves as a loading control.

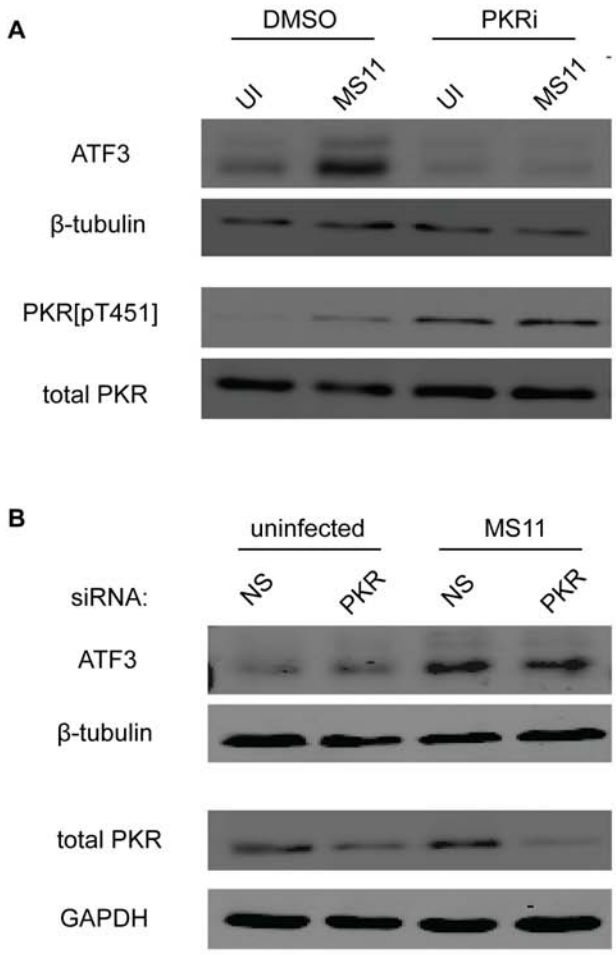

Figure 2-S2. PKR is phosphorylated by *N. gonorrhoeae* infection, but not required to induce ATF3 upregulation. (A) Treatment with a PKR inhibitor blocks gonococcal-induced ATF3 upregulation, but not PKR phosphorylation. T84 cells were treated with DMSO or 5 μM of a chemical inhibitor of PKR (PKRi) for one hour prior to infection with MS11. Cell lysates were harvested at three hours post-infection and ATF3 levels assessed by western blot. (B) Knockdown of endogenous PKR by RNA interference. T84 cells were nucleofected with nonsense (NS) or PKR-specific (PKR) siRNA. At 72 hours post-infection, the cells were infected with MS11 for three hours and protein levels assessed by western blot.

Chapter 3: Manuscript 2

Induction of the Innate Immune Response by the Commensal Species *Neisseria elongata*

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Introduction

The *Neisseria* are a large group of gram-negative bacteria that infect mucosal epithelial surfaces [3]. Of these, only two are pathogenic for humans: *Neisseria meningitidis*, a cause of bacterial meningitis and septicemia, and *Neisseria gonorrhoeae*, the etiological agent of gonorrhea. Despite their association with disease, both *N. meningitidis* and *N. gonorrhoeae* can exist asymptomatically in the host. Up to 35% of healthy individuals carry *N. meningitidis* with no overt symptoms of disease [9] and carriage can last for up to five months [10]. Over five percent of the general population between 16 and 35 years of age carry *N. gonorrhoeae* asymptomatically [13]. Asymptomatic gonorrhea is of particular concern for women; approximately 60% of women with cervical gonococcal infections do not show obvious symptoms of disease [2]. Left untreated, gonorrhea can lead to pelvic inflammatory disease, ectopic pregnancy and infertility [19]. The strategies that *N. gonorrhoeae* employs to induce asymptomatic infection are not well understood.

At least eight species of commensal *Neisseria* can also infect human mucosal surfaces [3]. Commensal *Neisseria* are part of the normal flora of nasopharynx. However, commensals can be isolated from other mucosal epithelial surfaces, including the urogenital tract [129-131]. Similarly, *N. gonorrhoeae* can be isolated from non-urogenital sites such as nasopharyngeal mucosal surfaces [15]. Thus commensal and pathogenic *Neisseria* can occupy similar niches in humans.

Unlike the pathogens, commensal *Neisseria* are poorly characterized, and little is known about how they colonize and persist in man. A comprehensive genetic analysis of the human commensal *Neisseria* was recently completed; this study revealed that the two pathogenic species have evolved from the commensals [28]. Moreover, commensals

encode a large number of virulence genes, including a complete set of type IV pilus (Tfp) biogenesis genes. Tfp are fimbriate structures with multiple functions, including DNA uptake, twitching motility, microcolony formation and attachment to host tissues [132]. Tfp also influence host responses to infection, including the expression of immune response genes [93], down-regulation of apoptosis [93-95], and processing of the immunoregulatory protein CD46 [90]. Recent work has determined that *Neisseria elongata*, the most basal species on the *Neisseria* phylogenetic tree, produces Tfp that function in DNA transformation and attachment and microcolony formation [29]. Thus, Tfp is an ancestral *Neisseria* trait that has been inherited by the pathogens. Given the role of Tfp in modulating the host response to *N. gonorrhoeae* infection, it is reasonable to postulate that commensal *Neisseria* may also use Tfp to modify host immune responses.

The mucosal epithelium represents the first line of defense against most bacteria. For many infections, a localized inflammatory response occurs at the initial site of infection, including the secretion of cytokines that promote recruitment of immune cells [44]. Both commensals and pathogens can induce innate immune responses; subtle differences between the two types of bacteria help determine whether these interactions result in asymptomatic colonization or disease. *N. gonorrhoeae* has inherited certain host adaptation factors, such as Tfp, from its commensal ancestors; expression of these factors may help explain the high rates of asymptomatic gonococcal infection. To begin examining how commensals modulate the host response, we tested the hypothesis that *N. elongata* can activate the innate immune system. Our findings suggest that *N. elongata* may induce the production of pro-inflammatory cytokines by human epithelial cells. *N. elongata* infection also increases NFκB phosphorylation and upregulates

expression of ATF3, a transcription factor that can negatively regulate the innate immune response. The host responses induced by *N. elongata* are consistently lower in magnitude than those induced by *N. gonorrhoeae*.

Results

N. elongata stimulates cytokine secretion by mucosal epithelial cells

To compare activation of the innate immune response by pathogenic and commensal *Neisseria*, we infected human epithelial cells with wild type strains of *N. gonorrhoeae* (MS11) or *N. elongata* (29315) and assessed cytokine secretion using a bead-based immunoassay. Two different epithelial cell lines were tested: an endocervical line (End1) that is representative of the urogenital cells from which *N. gonorrhoeae* is frequently isolated, and a nasopharyngeal line (Detroit 562) that is similar to the environment that *N. elongata* usually inhabits. Preliminary results suggest that *N. elongata* is capable of inducing the production of several cytokines and chemokines, including GM-CSF, GRO, IL-6, IL-8, and TNFα, but to a lesser extent than the levels seen with *N. gonorrhoeae* (data not shown). We are currently completing a more comprehensive study of the cytokine response to *N. elongata* infection. In collaboration with the Duke University Human Vaccine Institute, we will compare the levels of 30 different cytokines produced in human epithelial cells infected with either *N. elongata* or *N. gonorrhoeae*.

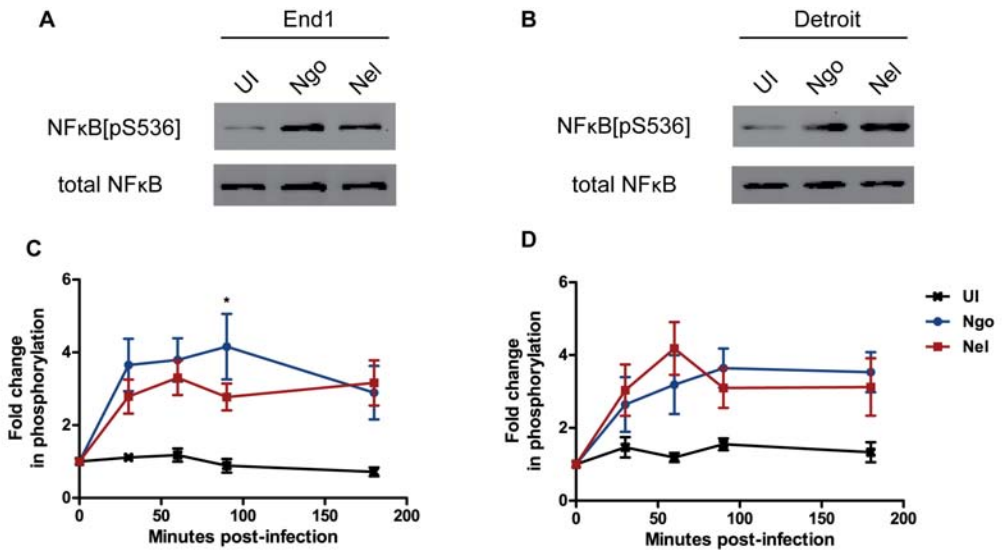

Figure 3-1. *N. elongata* infection induces NFκB phosphorylation. End1 cells (A) or Detroit cells (B) were infected with *N. gonorrhoeae* (Ngo) or *N. elongata* (Nel) at an MOI of 50 for one hour. NFκB activation was assessed by probing western blots with an antibody specific for the NFκB protein p65 phosphorylated at Ser536. Total NFκB was used as a loading control. (C-D) Densitometry anaylasis of NFκB phosphorylation. Samples were collected at the indicated time points from infected End1 (C) or Detroit (D) cells, subjected to western blot, and relative levels of p65 phosphorylation were assessed by densitometry. Values represent mean normalized NFκB[pS536] levels (±SEM, n=4). An asterisk denotes a *p* value of <0.05.

N. elongata induces NFκB phosphorylation

The transcription factor NFκB plays an important role in upregulating cytokine expression in response to a variety of pathogens, including *N. gonorrhoeae* [48, 151]. We hypothesized that the lower levels of cytokine secretion observed in *N. elongata*-infected cells were due to decreased activation of NFκB. To test this hypothesis, lysates from cells infected with *N. gonorrhoeae* or *N. elongata* were probed with an antibody specific for the NFκB protein p65 phosphorylated at Ser536. Phosphorylation at this residue is associated with enhanced transactivation activity by p65 [152]. *N. elongata* and *N. gonorrhoeae* induced NFκB phosphorylation in both endocervical and nasopharyngeal epithelial cells (Figure 3-1A and 3-1B). *N. gonorrhoeae*-infected End1 cells had slightly higher levels of phospho-NFκB compared to *N. elongata*-infected cells;

this difference was statistically significant at 90 minutes post-infection (Figure 3-1C). However, in Detroit cells, commensal and pathogen induced similar levels of NFκB phosphorylation throughout the time course of infection (Figure 3-1D). These results suggest that both *N. elongata* and *N. gonorrhoeae* trigger NFκB phosphorylation at Ser536 in two epithelial cell types. While *N. elongata* induced a slightly lower level of NFκB phosphorylation than *N. gonorrhoeae*, this difference is unlikely to account for the lower levels of cytokine production in *N. elongata*-infected cells.

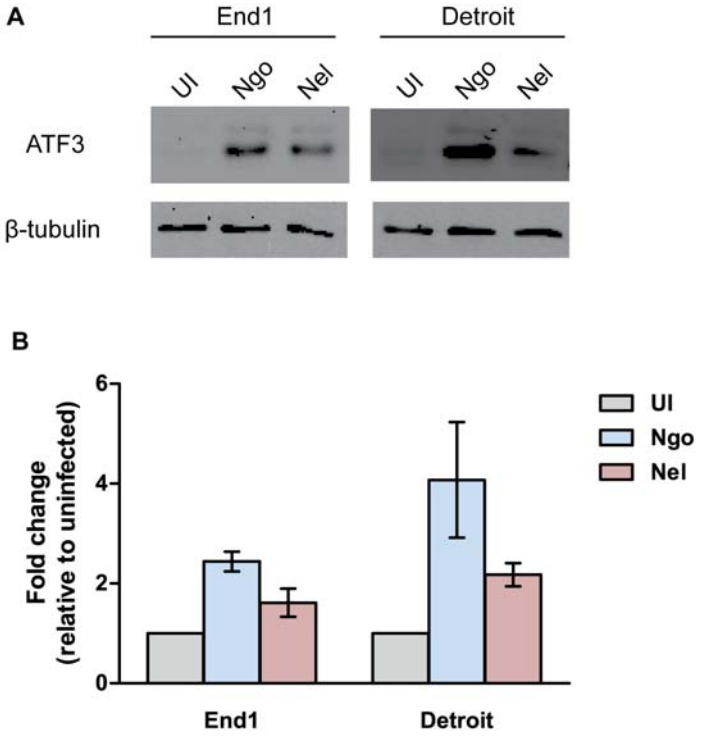

Figure 3-2. ATF3 is upregulated by *N. elongata* infection. (A) End1 cells or Detroit cells were infected with *N. gonorrhoeae* (Ngo) or *N. elongata* (Nel) for three hours and relative ATF3 levels assessed by western blot. Total β-tubulin serves as a loading control. (C) Densitometry values represent mean normalized ATF3 protein levels (±SEM, n=3 for End1 cells, n=2 for Detroit cells).

ATF3 is upregulated by *N. elongata* infection

The inflammatory response provides important protection from invading microbes; however, excessive inflammation can be damaging. To avoid chronic inflammation, the host must therefore maintain mechanisms to dampen aspects of the immune response. The transcription factor ATF3 is an important negative regulator of both innate and adaptive immune responses [101]. We have previously demonstrated that *N. gonorrhoeae* upregulates expression of ATF3, and that this expression is important for repressing IL-6 transcription during gonococcal infection (see Chapter 2). To determine whether *N. elongata* can induce ATF3 expression, we assessed ATF3 protein levels of infected epithelial cells by western blotting. Infection with *N. gonorrhoeae* served as a positive control. We found that *N. elongata* also induces expression of ATF3, although at reduced levels compared to *N. gonorrhoeae* in both End1 and Detroit epithelial cells (Figure 3-3).

Discussion

The preliminary results of our study suggest that *N. elongata* can induce secretion of pro-inflammatory cytokines from infected epithelial cells (data not shown). Activation of the host immune response has been observed previously with the commensal species *Neisseria lactamica*. Infection with *N. lactamica* induces lower levels of cytokine production in nasopharyngeal and meningeal cells when compared to *N. meningitidis* [153, 154]. Furthermore, pre-incubating nasopharyngeal cells with *N. lactamica* reduces the amounts of cytokine induced by *N. meningitidis* infection. These findings suggest that commensal *Neisseria* can activate cytoprotective pathways in epithelial cells.

Our results strongly suggest that both commensal and pathogenic trigger the phosphorylation of NFκB at Ser536. *N. elongata*- and *N. gonorrhoeae*-infected Detroit cells have similar levels of phospho-NFκB. However, *N. gonorrhoeae*-infected End1 cells consistently had slightly higher levels of phospho-NFκB, particularly at 90 minutes post-infection. The slightly elevated levels of phospho-NFκB in *N. gonorrhoeae*-infected End1 cells may be due to tissue tropism, as *N. gonorrhoeae* is more commonly cultured from the urogenital tract than *N. elongata*. Our results are consistent with studies comparing the abilies of the pathogen *N. meningitidis* and the commensal *N. lactamica* to induce IκBα degradation. IκBα binds to and inhibits NFκB activity by keeping the transcription factor sequestered in the cytosol [151]. Stimulation of the NFκB pathway leads to phosphorylation and degradation of the IκB complex, freeing NFκB to translocate to the nucleus and induce transcription. Thus, IκBα degradation is associated with increased NFκB activity. *N. meningitidis* and *N. lactamica* are identical in their ability to trigger IκBα phosphorylation and degradation in Detroit cells [153]. However, NFκB p65 isolated from *N. meningitidis*-infected cells binds DNA at significantly higher levels compared to p65 from *N. lactamica*-infected cells. These studies suggest that measuring phosphorylation levels may not be a sufficient indicator of NFκB activation by *Neisseria*. In future experiments I will compare NFκB activation by commensal and pathogen more directly by measuring the DNA binding capacity of p65 isolated from infected cells. If the levels of NFκB activation are lower in *N. elongata*-infected cells, we will also explore the mechanisms that this commensal employs to reduce NFκB activity. For example, Tezera et al. demonstrate that the cytoprotective effects of *N. lactamica* infection are mediated in part by upregulating the expression of peroxisome proliferator-activated receptor γ (PPARγ), a nuclear receptor that exports NFκB to the cytosol [153].

A successful commensal must be able to establish a foothold in its host while causing minimal damage. We have presented evidence that *N. elongata* infection produces an attenuated innate immune response in mucosal epithelial cells. These results will provide the groundwork for future studies on how *N. elongata* dampens the host response to infection. Tfp retraction plays an important role in modifying the host response to infection with *N. gonorrhoeae* [93-95]. Therefore, we plan to test whether pilus retraction modulates the innate immune response during *N. elongata* infection as well. We are also interested in studying the evolution of host response to different *Neisseria* species. Thus far, we examined the host response to *N. elongata*, the most basal of the human *Neisseria* species (see Figure 1-2). Additional studies have utilized *N. lactamica*, the commensal most closely related to the pathogens. Examining the host immune response to other commensal *Neisseria* species will provide us with insights as to how interactions between *Neisseria* and epithelial cells have evolved. Combined with the preliminary data in this chapter, the planned studies will be summarized in a future manuscript. Ultimately our work with *N. elongata* should give new insights on the strategies that all *Neisseria* species, both commensal and pathogenic, utilize to establish asymptomatic infections in humans.

Materials and Methods

Reagents

The following antibodies were used in this study: anti-ATF3 sc-188 and anti-NFκB sc-8008 (Santa Cruz Biotechnology), anti-phospho-NFκB (Ser536, Cell Signaling) and anti-β-tubulin (E7, University of Iowa Developmental Studies Hybridoma Bank).

Tissue culture

Detroit 562 nasopharyngeal epithelial cells (ATCC #CCL-138) were maintained at 37°C with 5% CO_2 in Modified Eagle medium supplemented with non-essential amino acids and 10% heat-inactivated FBS. End1 endocervical epithelial cells (a gift from Deborah J. Anderson) were grown in EpiLife (Invitrogen) containing human keratinocyte growth supplement (0.2% v/v bovine pituitary extract, 5 mg/ml bovine insulin, 0.18 mg/ml hydrocortisone, 5 mg/ml bovine transferrin, 0.2 ng/ml human epidermal growth factor), and 0.4 mM calcium chloride. Cells were passaged every four to five days and only used up to a passage number of 12 (Detroit cells) or 72 (End1 cells). For all experiments, cells were seeded into 6-well tissue culture plates and used at approximately 80-90% confluency.

Bacterial strains and infections

The *N. elongata* subsp. *glycolytica* strain 29315 (ATCC) and the *N. gonorrhoeae* strain MS11 were used for all experiments. Strains were grown on gonoccocal medium base (GCB) agar (Difco) with Kellogg's supplements I and II at 37°C with 5% CO_2. Piliation and Opa phenotypes were determined by observing colony morphology using light microscopy. Only piliated, Opa non-expressing bacteria were used. For infections, 18-hour-old bacteria were swabbed from GCB agar and resuspended in liquid GCB medium. Epithelial cells were infected with bacteria at a multiplicity of infection (MOI) of 50, or mock infected with GCB liquid medium.

Cytokine Immunoassays

Spent media from mock- or *Neisseria*-infected cells were collected at six hours post-infection and centrifuged at 16,100 × *g* for five minutes at 4°C. Clarified supernatants were stored at -80°C until further processing. Cytokine concentrations were determined using the Milliplex MAP immunoassay (Miilipore), according to the manufacturer's instructions.

Preparation of protein lysates

For ATF3 samples, cells were washed two times with ice cold PBS and then lysed in ice cold RIPA buffer (10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1% w/v SDS, 1% deoxycholate, 1% v/v Triton X-100) supplemented with 1X Complete, EDTA-free protease inhibitor cocktail (Roche) and 1X PhosSTOP phosphatase inhibitor cocktail (Roche). Samples were incubated on ice for 20 minutes before being scraped into microfuge tubes. Lysates were clarified by centrifugation at 16,100 × *g* for 20 minutes at 4°C. For NFκB samples, cells were lysed in 1X SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% v/v glycerol, 50 mM DTT, 0.1% w/v bromophenol blue) supplemented with protease and phosphatase inhibitors. The lysates were each run through a 23 gauge needle to shear the DNA. All samples were store at -80°C until further processing.

Immunoblotting

Protein samples were resolved by SDS-PAGE and transferred onto a 0.45 μm nitrocellulose membrane (GE Osmonics). Blots were blocked for one hour in Tris-buffered saline (TBS) containing 0.1% Tween and 5% nonfat dry powdered milk. Primary

antibodies were diluted in either 5% milk/TBST (ATF3, β-tubulin) or 5% BSA/TBST (phospho-NFκB) and incubated overnight at 4°C. The blots were imaged on the LICOR Odyssey Infrared Imaging System, and band intensities were measured by densitometry using Image J v1.36b.

Statistics

Statistical analyses were performed using Prism 5 (GraphPad Software). Significant differences were determined by one-way ANOVA, followed by Bonferroni's post hoc comparison test.

Acknowledgments

We would like to thank Claire Larmonier and Pawel Kiela for assistance with the cytokine arrays, and María Rendón for help optimizing *N. elongata* infection conditions.

Chapter 4:

Discussion and Conclusions

I. Review of Dissertation

Both pathogenic and commensal *Neisseria* can establish asymptomatic infections in the mucosal epithelia, suggesting that they have developed strategies to modulate host defense mechanisms. In this dissertation, I tested the hypothesis that *Neisseria* species modulate the innate immune response by inducing expression of the transcriptional regulator ATF3. I presented evidence that *N. gonorrhoeae* upregulates ATF3 expression during infection of epithelial cells; in turn, ATF3 negatively regulates IL-6 expression. In the introductory chapter, I described the genomic sequencing and comparison studies of the *Neisseria* genus, and the significance of the findings to our understanding of the behaviors of commensal and pathogenic *Neissieria*. These findings led me to test whether commensal *Neisseria* can activate the host immune response. I found that N. elongata can also activate the innate immune response and induce ATF3 expression. In this chapter, I will discuss the relevance of these cumulative findings to *Neisseria* biology. In particular, I will discuss the host cell signaling pathways involved in ATF3 upregulation and other potential targets of ATF3 transcriptional regulation. I will also discuss cytokine induction during *Neisseria* infection, and the cytoprotective pathways activated by commensals. I conclude by presenting a model for the role of ATF3 in *Neisseria* infection.

II. Signaling Pathways Involved in ATF3 Upregulation

Tfp-Activated MAPK Signaling

Epithelial cells infected with Tfp retraction-deficient Δ*pilT* mutants have lower levels of ATF3 when compared to cells infected with the wild type parent strain (Figure 2-1). This indicates that Tfp retraction enhances expression of ATF3. ATF3 expression is also

induced at lower levels in cells infected with the commensal species *N. elongata*. There are two possible explanations to account for this difference in ATF3 regulation by commensal and pathogen. The first, and most simple, explanation is that *N. elongata* produces fewer Tfp than *N. gonorrhoeae* [29]. Alternatively, the difference in the ability of commensal and pathogen to upregulate ATF3 may be due to the mechanical forces their Tfp exert on the epithelial cell. Our collaborator, N. Biais, has gathered compelling data that *N. elongata* Tfp retracts with less force than *N. gonorrhoeae* Tfp (N. Biais, manuscript in preparation). Our lab has previously shown that Tfp retraction force, *per se*, stimulates the MAPK signaling pathway [93]. As discussed in Chapter 2, MAPK activation is required for *N. gonorrhoeae*-induced ATF3 expression (Figure 2-3). These studies establish a link between mechanical force on the epithelial cell surface and a signaling pathway upstream of ATF3 expression. These explanations are nonexclusive; the ultimate cause of the differential upregulation of ATF3 by commensal and pathogen may be a combination of the two.

Collectively, our data demonstrate that Tfp retraction activates MAPK signaling pathways in the host cell, which in turn lead to increased expression of ATF3. However, cells infected with a *pilT* mutant still produce low levels of ATF3, suggesting that *Neisseria* can also upregulate ATF3 in a retraction-independent manner. I hypothesize that gonococcal MAMPs may upregulate ATF3 expression by stimulating TLR activation.

Toll Receptor Signaling

Toll receptors detect infection by binding molecular patterns that are commonly expressed by microbes and then activating signaling cascades that control the innate immune response [119]. Purified bacterial components such as LPS can induce ATF3

expression by activating TLR pathways [120-122]. I have demonstrated that treating epithelial cells with crude outer membrane preparations from *N. gonorrhoeae* upregulates ATF3. This result indicates that bacterial components alone are sufficient to induce a basal level of ATF3 expression (Figure 2-2). Moreover, it suggests that one or more gonococcal outer membrane proteins may upregulate ATF3 expression via TLR activation. As *Neisseria* outer membrane proteins are known to activate TLR2 signaling [137, 138], I tested whether ATF3 upregulation could be inhibited by blocking TLR2 activation. Epithelial cells were incubated with a TLR2 neutralizing antibody prior to infection with wild type *N. gonorrhoeae*; however, no reduction in ATF3 levels was observed (data not shown). Previous attempts to block the innate immune response to *N. gonorrhoeae* by inhibiting a single Toll receptor have also been unsuccessful. Gonoococcal lysates were able to activate NFκB in cells pretreated with a TLR2 neutralizing antibody, or in cells expressing a TLR2 dominant-negative construct [135]. Only a MyD88 dominant-negative construct, which completely inhibited TLR signaling, succeeded in reducing NFκB activation by the gonococcal lysates. Together, these findings strongly suggest that *N. gonorrhoeae* activates host responses, including ATF3 expression, through multiple TLR pathways. Future experiments to test whether TLR signaling is required for ATF3 expression during *N. gonorrhoeae* infection will include the use of cells transiently transfected with a MyD88 dominant-negative construct.

III. Additional Targets of *N. gonorrhoeae*-induced ATF3 Regulation

Epithelial Cell Targets

Chromatin immunoprecipitation and microarray analyses have identified over 1000 genes whose promoters contain ATF3 binding sites [118]. Of these genes, at least 30 have been verified as targets of ATF3 transcriptional regulation [101, 155]. Thus, it is reasonable to hypothesize that ATF3 in *N. gonorrhoeae*-infected cells will regulate the expression of genes in addition to IL-6. Among the genes known to be regulated by ATF3 in epithelial cells, a few stand out as potential targets. Expression of cyclin D1, which regulates progression of the cell cycle from G1 to S phase, is repressed by ATF3 in fibroblasts [156]. Interestingly, cyclin D1 is downregulated in endocervical cells infected with *N. gonorrhoeae* [157]. Given that *N. gonorrhoeae* causes G1 arrest during infection, it is reasonable to speculate that downregulation of cyclin D1 by ATF3 plays a role in this process.

Although ATF3 generally acts as a negative regulator of transcription, it has been shown to upregulate the expression of a handful of genes. For example, ATF3 can upregulate expression of the cytokines IL-1β and TGF-β [158, 159]. IL-1β expression is higher in human male volunteers infected with gonorrhea [16] and IL-1β and TGF-β transcripts are elevated in gonococcal-infected endocervical tissue [48]. These results suggest that upregulation of ATF3 by *N. gonorrhoeae* may induce the expression of at least two cytokines.

ATF3 also induces expression of *FN-1*, which encodes the extracellular matrix glycoprotein fibronectin [160]. Fibronectin serves as a receptor for the gonococcal outer

membrane protein OpaA, and is required for OpaA-mediated invasion of epithelial cells [161]. In light of these findings, it is tempting to speculate that *N. gonorrhoeae* upregulation of ATF3 leads to production of fibronectin, which in turn facilitates OpaA-mediated gonococcal binding to and invasion of the epithelial cell.

ATF3 Targets in Other Cell Types

Mucosal epithelial cells are the primary sites of infection for *N. gonorrhoeae*. However, during the course of infection, *N. gonorrhoeae* will interact with other cell types and can potentially induce ATF3 expression in them. Notably, *N. gonorrhoeae* will encounter different leukocytes that are recruited to the site of a productive infection. Human polymorphonuclear leukocytes challenged with *N. gonorrhoeae* secrete IL-6 and TNFα [162]; peripheral blood mononuclear cells infected with *N. gonorrhoeae* produce IFNγ, IL-4, IL-6, IL-10, IL12, and TNFα [163]. All of these cytokines are known targets of ATF3 regulation [101, 155].

IV. Cytokine Induction by *Neisseria*

N. gonorrhoeae induces the secretion of several pro-inflammatory cytokines from infected human tissues [16, 45-50]. From the perspective of the host, the inflammatory response is advantageous, as it promotes clearance of the infecting bacteria. However, increased cytokine expression may also benefit *N. gonorrhoeae*. It has been proposed that tissue damage from the inflammatory response could increase access of the bacteria to subepithelial compartments, facilitating its dissemination to other sites in the body [138]. However, disseminating gonococcal infections are rare; moreover, such a

debilitating infection would inhibit the type of behavior (i.e. sexual contact) that is required for transmission of the bacterium. Thus it seems unlikely that *N. gonorrhoeae* would upregulate cytokine expression to facilitate invasion of deeper tissues.

An alternative hypothesis is that transient expression of certain cytokines may be beneficial for the infecting organism. As discussed in Chapter 2, IL-6 induces expression of anti-apoptotic factors [149]. Thus, transient expression of IL-6 in infected tissues may help *N. gonorrhoeae* establish infection by preventing epithelial cell death. IL-6 production is also required to induce differentiation of T helper 17 (Th17) cells [164]. The Th17 response functions in part by recruiting neutrophils to the site of infection. *N. gonorrhoeae* has been shown to induce Th17 cell differentiation and neutrophil recruitment in a mouse model of infection [165, 166] Interestingly, *N. gonorrhoeae* can survive and replicate within neutrophils [167, 168]; indeed, a purulent discharge containing neutrophils and gonococci is a hallmark of gonorrhea infection [15]. Based on these observations, it has been proposed that *N. gonorrhoeae* preferentially activates Th17 cells to skew the immune response in its favor, by recruiting a cell type (neutrophils) in which gonococci have adapted to survive [169]. In support of this model, men with natural or experimental gonococcal infections exhibit increased levels of Th17-associated cytokines, including IL-6, IL-17A, and IL-23 [16, 170].

V. Activation of Cytoprotective Pathways by Commensal *Neisseria*

Cytoprotective Signaling Cascades

Commensals are by definition bacteria that infect their hosts without causing damage [171]. Indeed, many commensal species activate cytoprotective pathways that help

minimize host cell damage [172]. In Chapter 3, I presented data demonstrating that *N. elongata* infection upregulates expression of ATF3 (Figure 3-2), which confers cytoprotective effects by repressing the inflammatory response. Preliminary data also indicate that *N. elongata* can activate MAPK signaling in infected epithelial cells (data not shown). As MAPK activation is required for ATF3 expression during *N. gonorrhoeae* infection (Figure 2-3), it reasonable to hypothesize that induction of ATF3 by commensal *Neisseria* would be regulated in a similar manner.

Preliminary experiments in our lab also suggest that commensal *Neisseria* can activate the PI3K pathway, a cell signaling cascade that can promote cell survival [173]. PI3K activation is elevated in bronchial epithelial cells infected with *N. elongata*. Furthermore, treating cells with a chemical inhibitor of PI3K activity results in increased levels of apoptosis in *N. elongata*-infected cells (D. Higashi and So, unpublished results). Thus activation of PI3K by *N. elongata* may promote colonization by preventing host cell apoptosis.

Downregulation of the Immune Response

The observation that *N. elongata* induces lower levels of cytokine production suggests that commensals can activate cytoprotective pathways that inhibit activation of the immune response. This hypothesis is supported by recent work on the commensal *N. lactamica*. Pre-incubating nasopharyngeal cells with *N. lactamica* can lower the levels of IL-6, IL-8, and TNFα induced by a subsequent *N. meningitidis* infection. *N. lactamica*-induced cytoprotection was mediated by increased expression of PPARγ [153]. PPARγ is a nuclear receptor that can complex with NFκB p65 and facilitate its export from the

nucleus [174]. PPARγ expression reduces inflammation in blood vessel endothelial cells, during ischemia/reperfusion-induced injury, and in gut epithelial cells cultured with the commensal microbe *Bacteroides thetaiotaomicron* [174-176]. Whether *N. elongata* infection also upregulates PPARγ expression has yet to be tested.

VI. Proposed Model for ATF3 Function During *Neisseria* Infection

Based on the findings of my thesis and previous reports, I propose a model for the role of ATF3 in *Neisseria* infection (Figure 4-1). *Neisseria* infection can activate host cell signaling via two pathways: a Tfp retraction-dependent pathway (step 1) or a retraction-independent pathway (step 2). Activation of the retraction-independent pathway, which is hypothesized to involve TLR stimulation by neisserial MAMPs, upregulates expression of host stress response genes like IL-6 and ATF3. IL-6 expression is upregulated at the earliest time points of infection [48], while ATF3 upregulation occurs slightly later, begininning at approximately three hours post-infection. Tfp retraction enhances activation of signaling pathways, including the MAPK. Greater MAPK activation due to pilus retraction leads to enhanced expression of ATF3 and IL-6 (step 3). Upregulation of IL-6 leads to increased secretion of this cytokine, which helps recruit PMNs to the site of infection (step 4). IL-6 may also induce expression of the pro-survival protein MCL-1, which would facilitate infection by preventing epithelial cell death (step 5). Expression of ATF3 leads to transcriptional regulation of its target genes. ATF3 can upregulate expression of the cytokines IL-1β and TGF-β (step 6). ATF3 may also induce expression of fibronectin, an extracellular matrix protein that facilitates *N. gonorrhoeae* invasion. ATF3 also negatively regulates the transcription of genes (step 7). ATF3 is required to

suppress IL-6 expression during gonococcal infection. Another potential ATF3 target is cyclin D1, which is known to be downregulated by *N. gonorrhoeae* and is required for progression of the cell cycle. Future experiments will help define the pathways that ATF3 participates in during infection.

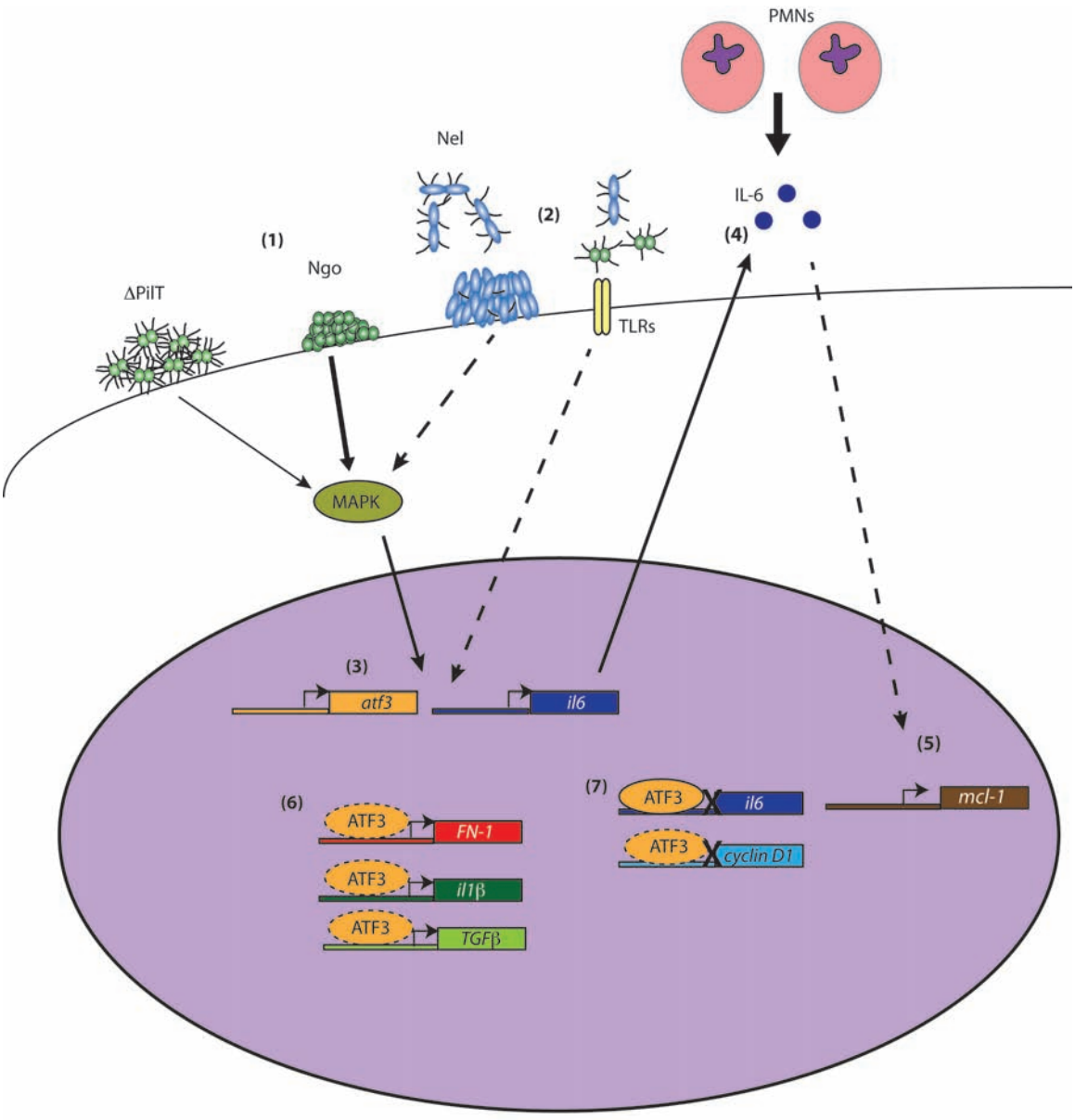

Figure 4-1. Model for the role of ATF3 during *Neisseria* infection. *Neisseria* infection can activate cell signaling cascades via a Tfp retraction-dependent pathway (1), or a retraction-independent pathway (2) that may involve TLR signaling. Retraction-dependent signaling activates MAPK pathways, which enhance gene expression. Expression of ATF3 and IL-6 (3) are induced by both retraction-dependent and retraction-independent pathways. Secreted IL-6 (4) can recruit PMNs to the site of infection, or induce expression of the anti-apoptotic protein MCL-1 in epithelial cells (5). ATF3 protein can induce expression of FN-1, IL-1β, and TGF-β (6) or repress expression of IL-6 and cyclin D1 (7). Solid lines represent events shown to occur in this thesis or previous work. Dashed lines represent hypothesized signaling events or actions. Heavier lines denote stronger signaling events in response to Tfp retraction. See text for additional details.

Taken in perspective with the *Neisseria* phylogenetic tree (Figure 1-2), my studies strongly suggest that ATF3 upregulation is a trait that was evolved by the earliest commensal *Neisseria* species to dampen the host inflammatory response to infection; this trait has been retained by the pathogen *N. gonorrhoeae* during its evolution (Figure 3-3). In support of this notion, commensal *Neisseria* infection has been shown to induce a comparatively mild inflammatory response (data not shown) [153, 154], and upregulation of ATF3 would quell the innate immune response and facilitate asymptomatic infection. *N. gonorrhoeae* encodes only a handful of pathogen-specific genes, several of which are essential for survival in humans [28]. One pathogen-specific gene encodes an IgA protease that cleaves human IgA1 as well as the lysosomal membrane protein LAMP1 [72, 177, 178]. Purified IgA protease is a potent inducer of pro-inflammatory cytokines and T cell activation [179, 180]. Thus, the increased expression of ATF3 seen during gonococcal infection may be required to inhibit the stronger inflammatory response that *N. gonorrhoeae* infection elicits.

VII. Conclusions

In order to establish and maintain an asymptomatic state in its host, *N. gonorrhoeae* has developed strategies to modulate host defense mechanisms. Previous work has established that *N. gonorrhoeae* can activate cytoprotective pathways in the cell, including the MAPK and PI3K signaling cascades, that downregulate apoptosis [63, 93-95, 181, 182]. In this dissertation, I presented evidence that *N. gonorrhoeae* can also induce cytoprotection via ATF3, a transcriptional regulator of the innate immune response. ATF3 is upregulated by *N. gonorrhoeae* infection, and its expression is required to repress transcription of the pro-inflammatory cytokine IL-6. As ATF3 is also

upregulated by *N. elongata*, our findings suggest that ATF3 may represent a host cell pathway that is commonly exploited by both commensal and pathogenic *Neisseria* to establish asymptomatic infection in humans.

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