THE ROLE OF LYtic TRANSGlycosyLASES LTGA AND LTGD IN INNATE IMMUNE RECOGNITION AND PATHOGENESIS OF Neisseria gonorrhoeae

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ABSTRACT

Kayla Jean Knilans: The role of lytic transglycosylases LtgA and LtgD in innate immune recognition and pathogenesis of Neisseria gonorrhoeae (Under the direction of Joseph A. Duncan)

During growth, Neisseria gonorrhoeae releases inflammatory 1,6-anhydro peptidoglycan monomers through the action of lytic transglycosylases LtgA and LtgD. N. gonorrhoeae lacking LtgA and LtgD release peptidoglycan multimers rather than monomers. Because peptidoglycan is a known activator of the innate immune system, we sought to understand how the activity of LtgA and LtgD influenced host responses to N. gonorrhoeae. We tested inflammatory responses to N. gonorrhoeae using isolate FA1090 and FA1090 with ltgA and ltgD deleted, FA1090 ΔltgA/ΔltgD. Both live N. gonorrhoeae and culture supernatants from FA1090 ΔltgA/ΔltgD caused increased production of IL-1β and TNF-α in THP1 cells and primary human blood dendritic cells. Culture supernatants from FA1090 ΔltgA/ΔltgD, which contain multimeric peptidoglycan fragments, were more potent activators of host NOD2 and TLR2, but not TLR4, TLR9, or NOD1. NOD1 was activated equally by peptidoglycan monomers and multimers from N. gonorrhoeae, but NOD2 was activated only by multimers. To explain this, we showed that multimeric peptidoglycan digested by LtgA, which produce anhydro monomers, were poor stimulators of NOD2, while reducing peptidoglycan monomers produced by host lysozyme were potent stimulators of NOD2. Increased TLR2 activation in response to FA1090 ΔltgA/ΔltgD culture supernatants was not the result of activation by peptidoglycan, but the presence of higher
levels of TLR2-activating proteins. These data indicate that LtgA and LtgD allow *N. gonorrhoeae* to evade detection by host TLR2 and NOD2.

We next tested the roles of NOD2 as well as LtgA and LtgD in *N. gonorrhoeae* infection *in vivo*. *Nod2*−/− mice infected with FA1090 had similar bacterial burdens and persistence of infection as wild type mice, confirming *in vitro* data that wild type *N. gonorrhoeae* interacts minimally with NOD2. When wild type mice were infected with FA1090 ΔltgA/ΔltgD we observed a significantly lower bacterial burden compared to mice infected with FA1090. Competitive co-infection with FA1090 revealed a fitness defect for FA1090 ΔltgA/ΔltgD. There was not difference in infection persistence between FA1090 and FA1090 ΔltgA/ΔltgD, though less mice were successfully infected with FA1090 ΔltgA/ΔltgD than wild type FA1090. Overall our data is supportive of an important role for LtgA and LtgD in pathogenesis.
To my parents, who have always encouraged and supported me, and especially my dad, who introduced me to *Star Trek* and the idea that through science and diversity the world can be a better place.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>BMDC</td>
<td>bone marrow derived dendritic cells</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control</td>
</tr>
<tr>
<td>CEACAM</td>
<td>carcinoembryonic antigen cell adhesion molecules</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming units</td>
</tr>
<tr>
<td>CI</td>
<td>competitive index</td>
</tr>
<tr>
<td>iE-DAP</td>
<td>D-glutamyl-meso-diaminopimelic acid</td>
</tr>
<tr>
<td>GGI</td>
<td>gonoccocal genetic island</td>
</tr>
<tr>
<td>LLD</td>
<td>lower limit of detection</td>
</tr>
<tr>
<td>LOS</td>
<td>lipooligosaccharide</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MDP</td>
<td>muramyl dipeptide</td>
</tr>
<tr>
<td>NLR</td>
<td>nucleotide-binding oligomerization domain and leucine-rich repeat containing receptors</td>
</tr>
<tr>
<td>NOD1</td>
<td>nucleotide-binding oligomerization domain-containing protein 1</td>
</tr>
<tr>
<td>NOD2</td>
<td>nucleotide-binding oligomerization domain-containing protein 2</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PEA</td>
<td>phosphoethanolamine</td>
</tr>
<tr>
<td>PGN</td>
<td>peptidoglycan</td>
</tr>
<tr>
<td>PGRP</td>
<td>peptidoglycan recognition proteins</td>
</tr>
<tr>
<td>PID</td>
<td>pelvic inflammatory disease</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
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Chapter 1

Introduction

1.1 Introduction

*Neisseria gonorrhoeae* is an obligate human pathogen responsible for the sexually transmitted infection gonorrhea. In the United States it is the second most commonly reported sexually transmitted infection to the Centers for Disease Control (CDC), with an estimated 820,000 new cases each year (Centers for Disease Control, 2012a). *N. gonorrhoeae* primarily infects the genital tract of both men and women. Left untreated, gonorrhea can cause complications including septic arthritis and sterility in both men and women, and pelvic inflammatory disease in women. In pregnant women with genital tract infection, mother to infant transmission can occur during birth, resulting in an infection of the eyes that can lead to blindness if left untreated (Laga et al., 1989). Infection with *N. gonorrhoeae* can also increase susceptibility to acquiring other sexually transmitted infections, including HIV (Kaul et al., 2008). In recent years, acquisition of antibiotic resistance by *N. gonorrhoeae* has made gonococcal infections difficult to treat and is emerging as a major public health problem. A better understanding of the mechanisms of *N. gonorrhoeae* infection pathogenesis is critical for the development of new therapeutic approaches, including vaccine development.

1.2 Epidemiology of infection and antibiotic resistance

There were 334,826 cases of gonorrhea reported to the CDC in 2012 (Centers for Disease Control, 2012b). The reported cases represent less than half of all estimated new cases, at least
partially because gonorrhea infection is often asymptomatic in women and occasionally asymptomatic in men. Approximately 60% of the cases reported in 2012 occurred in individuals aged 15-24 years. Within that age group, rates are slightly higher among women than men. Additionally, infection rates are highest among African-Americans than in other ethnically defined subgroups. Consistent with previous years, reported cases were highest in the southern United States.

A recent report from the CDC characterized antibiotic resistance in *N. gonorrhoeae* clinical isolates (Centers for Disease Control, 2013). There were an estimated 246,000 cases of antibiotic-resistant gonorrhea in the US in 2011. Based on the prevalence of cefixime resistance, the CDC estimated that there were 11,480 cases of cefixime-resistant *N. gonorrhoeae* infection. Due to the increase in cefixime-resistant cases, the CDC removed cefixime as a therapy for treating gonorrhea infection, leaving injectable ceftriaxone as the only first-line antibiotic for gonococcal infection (Centers for Disease Control, 2012c). *N. gonorrhoeae* resistance to these antibiotics is rising. In 2011, the CDC estimated 3,280 cases had reduced susceptibility to ceftriaxone, threatening the last remaining first-line agent for treatment of *N. gonorrhoeae*. Globally, the threat of drug-resistant *N. gonorrhoeae* is growing rapidly. A 2011 report described a *N. gonorrhoeae* isolate from a female sex worker in Japan, designated H041, with high ceftriaxone resistance (Ohnishi et al., 2011). A second strain isolated in France, F89, was also reported to have high resistance to cefixime and ceftriaxone (Unemo et al., 2012). Given that these antibiotics are currently the last remaining options for first-line treatment, there is the potential for the development of incurable gonorrhea.
1.3 Sites of infection and clinical manifestations

The most common site of infection is the genital tract. In women, 50-80% of infections are reported as being asymptomatic (Edwards and Apicella, 2004; Moran, 2007). If symptoms are present, they include increased vaginal discharge, dysuria, and uterine bleeding. In cases where initial physical examination appears normal, swabbing of the cervix may reveal purulent discharge (Curran et al., 1975). Around 45% of women with genital tract infection will develop ascending infection (Edwards and Apicella, 2004). This can lead to the development of pelvic inflammatory disease (PID), a serious complication of gonorrhea infection in women. PID is an often painful inflammation of the reproductive organs which can lead to tissue scarring, ectopic pregnancy, and infertility (Centers for Disease Control, 2014).

It is estimated that the risk of a male contracting urethral gonorrhea from an infected female partner is 20% for a single exposure (Holmes et al., 1970). Urethral gonorrhea infection in men is usually symptomatic. Infection is reported to be asymptomatic in men in 1-3% of cases (Harrison et al., 1979; Hook III and Handsfield, 2008), though in other reports the statistic is much higher (Handsfield et al., 1974). Discrepancies in these reports are likely the result of sampling bias, differences in the sensitivity of methods used to detect early infection, and whether or not the sexual partner through which transmission occurred had symptomatic infection. Studies in male human subjects have demonstrated that there is an incubation period between infection and the onset of symptoms of 48-96 hours (Schneider, 1991). The predominate symptoms experienced by men with *N. gonorrhoeae* infection are urethral discharge or dysuria (Hook III and Handsfield, 2008). It is estimated that prior to the availability of antibiotic therapy 95% of infections would resolve spontaneously within six months (Hook III
and Handsfield, 2008; Pelouze, 1941). As in women, serious complications of untreated infection can occur, most notably epididymitis (Watson, 1979).

A less common site of *N. gonorrhoeae* infection is the pharynx. In most reported studies, pharyngeal infections represent on average less than 10% of total gonorrhea infections, though rates tend to be higher in women (10-14%) and men who have sex with men (21%) (Odegaard and Gundersen, 1973; Wiesner et al., 1973). Pharyngeal infections are typically asymptomatic and most clear within 12 weeks (Wallin and Siegel, 1979). Pharyngeal infections that do not resolve require higher doses of antibiotics to reliably clear the bacteria than genital tract infection and may serve as a reservoir for antibiotic resistant *N. gonorrhoeae* (Hutt, 1986; Newman et al., 2007; Weinstock and Workowski, 2009).

Rectal infection occurs mostly commonly in women and men who have sex with men and, like pharyngeal infection, is commonly asymptomatic (Klein, 1977). Rectal infections in women can be the result of contamination with infected cervical secretions or receptive anal intercourse (Hunte et al., 2010; Kinghorn and Rashid, 1979). Rectal infections in men are almost always the result of receptive rectal intercourse (Hook III and Handsfield, 2008). Treatment success with antibiotic therapy is similar to that observed in cervical and urethral infections (Moran, 1995; Schroeter and Reynolds, 1972).

Disseminated gonococcal infection occurs in 0.5-3% of patients with untreated gonorrhea (Hook III and Handsfield, 2008). Disseminated infection is more common in women than men and some strains of *N. gonorrhoeae* are more likely to disseminate than others (Holmes et al., 1971). The most common symptoms of disseminated infection are tenosynovitis, skin lesions, and arthritis. One study of 49 patients with disseminated infection reported that 61% of patients had tenosynovitis, 71% had skin lesions, and 39% had arthritis (O’Brien et al., 1983). Treatment
with an effective antibiotic regimen can usually resolve these symptoms within 48 hours (Handsfield, 1976).

1.4 The immune response to *N. gonorrhoeae*

1.4.1 General innate sensing of bacterial pathogens

Recognition of bacterial pathogens by the immune system involves the cooperation of numerous intracellular and extracellular pattern recognition receptors (PRRs), each with specificity to particular pathogen-associated molecular patterns (PAMPs). PRR families include the extracellular toll-like receptors (TLR) and c-type lectin receptors (CLR), the intracellular NOD-like receptors (NLR), and the secreted peptidoglycan recognition proteins (PGRP).

The Toll proteins and their role in the immune response to pathogens was first described in *Drosophila melanogaster* (Lemaitre et al., 1996). To date, 10 functional human TLRs have been cloned (Chuang and Ulevitch, 2001, 2000; Du et al., 2000; Rock et al., 1998; Takeuchi et al., 1999a) and six of these—TLR1, TLR2, TLR4, TLR5, TLR6 and TLR9—recognize bacterial components. TLR2 can function alone or form heterodimers with TLR1 or TLR6 to recognize lipoproteins and lipopeptides of different structural specificities (Morr et al., 2002; Takeuchi et al., 2001, 2002). TLR4, in association with cofactor MD-2, recognizes bacterial lipopolysaccharide (LPS) or, in the case of some gram negative bacteria such as *N. gonorrhoeae*, lipooligosaccharide (LOS) (Shimazu et al., 1999). TLR5 recognizes bacterial flagellin (Hayashi et al., 2001) and, finally, TLR9 recognizes unmethylated CpG dinucleotides in bacterial DNA (Takeshita et al., 2001). Activation of these receptors initiates a signaling cascade that leads to the production of inflammatory cytokines which trigger a variety of responses including recruitment and differentiation of immune cells, antigen presentation, and programmed cell death (Akira and Takeda, 2004).
The NLR family of proteins is a diverse set of intracellular PRRs that can recognize both PAMPs and danger associated molecular patterns. The NOD1 and NOD2 proteins, for example, have both been well-described to recognize specific moieties of bacterial peptidoglycan (Franchi et al., 2009). Many NLRs form complexes known as inflammasomes, which trigger IL-1β maturation through the activation of caspase-1 (Martinon et al., 2002). The NLRP3 inflammasome has been shown to play a role in the innate immune response to bacterial infection by recognizing pore-forming toxins (Munoz-Planillo et al., 2009). The NLRP1 inflammasome is activated in the presence of muramyl dipeptide (MDP), the peptidoglycan ligand for NOD2, and has been shown to complex with NOD2 in response to Bacillus anthrasis infection and MDP stimulation (Faustin et al., 2007; Hsu et al., 2008). Inflammasome complexes containing NLR family members NLRC4 and NAIP51 or NAIP5 respond to bacterial flagellin and type III secretion systems (Halff et al., 2012; Miao et al., 2006, 2010; Yang et al., 2013). Many of the mechanisms that dictate NLR recognition of their ligands and their subsequent role in controlling bacterial infections are still unknown.

Additional proteins involved in the innate immune recognition of bacterial infections include the CLRs and PGRPs. The CLR superfamily is a diverse set of receptors that can be soluble proteins or expressed on the surface of many immune cell types, including macrophages and dendritic cells. They can mediate cell-cell interactions and some have been shown to bind the saccharide structures of bacterial cell walls (Weis et al., 1998). Finally, the four mammalian PGRPs are intracellular and secreted proteins that recognize bacterial peptidoglycan and also have bactericidal activity (Lu et al., 2005). The mammalian PGRPs are described in more detail in section 1.5.4
1.4.2 Characterization of the immune response to N. gonorrhoeae

Gonorrhea infection fails to induce a protective adaptive immune response in the host. The lack of a protective adaptive immune response to N. gonorrhoeae is mediated by a combination of mechanisms. Well-defined mechanisms of gonoccocal immune evasion include suppression of T-cell activation and proliferation, poor induction of broadly reactive anti-gonoccocal antibodies responses, and antigenic variation of bacterial surface components. Though the development of a protective adaptive immune response is suppressed in N. gonorrhoeae infection, inflammatory responses to colonization by the bacteria does occur in many cases, likely due to innate immune recognition. The key components of the immune response to N. gonorrhoeae discussed in this section are summarized in Figure 1.1.

The immune response to N. gonorrhoeae is typically characterized by the influx of polymorphonuclear leukocytes (PMNs). The purulent discharge characteristic of symptomatic infection in men and some women is the result of PMN influx and shedding of epithelial cells. Cytokines known to drive PMN migration to an infection site, IL-17A and IL-23, were found to be elevated in the serum of male patients with gonorrhea infection compared to uninfected controls (Gagliardi et al., 2011; Laan et al., 1999; Wu et al., 2007). Experimental infections have revealed the presence of IL-8, IL-6, and TNF-α within the urethra (Ramsey et al., 1995). In contrast, local increases of IL-1, IL-6, IL-8, and IL-10 in the genital secretions women with gonorrhea infection have not been observed, and serum levels of IL-6 in infected women were only modestly elevated (Hedges et al., 1998). Though localized cytokine production was not detected in clinical specimens from infected women, in vitro studies with immortalized vaginal and cervical epithelial cells report an increase in these cytokines upon stimulation with N. gonorrhoeae (Fichorova et al., 2001). The symptomatic status of the women the clinical samples
were analyzed from was not reported, but the lack of local increases in inflammatory cytokines may correlate with the asymptomatic gonorrhea infections typically observed in women.

Subjects with *N. gonorrhoeae* infection were found to have modestly increased levels of anti-gonococcal serum IgG in men and IgA1 in women compared to uninfected subjects, and prior infection with *N. gonorrhoeae* did not alter antibody levels in patients with current infection (Hedges et al., 1999). Locally, anti-gonococcal IgG and IgM antibodies do not appear to be significantly higher in the cervical mucus of infected and uninfected women. Levels of local anti-gonococcal antibodies in men were not analyzed in this study due to the low number of male patients with detectable antibody levels (Hedges et al., 1999). *In vitro*, *N. gonorrhoeae* has an ability to kill CEACAM1 expressing B cells and suppress antibody production (Pantelic et al., 2005). Recently, it was shown that B cells exposed to *N. gonorrhoeae* in culture produced non-specific IgM. Levels of anti-gonococcal antibodies produced by B cells exposed to *N. gonorrhoeae* were similar to the levels of antibodies against tetanus toxin, an antigen the subjects had likely been exposed to previously through vaccination, and Keyhole limpet hemocyanin, a control antigen that subjects should not have been exposed to previously (So et al., 2012). If this non-specific induction of antibody production occurs *in vivo*, the effect is likely to be further exacerbated by a lack of T-cell co-stimulation. *N. gonorrhoeae* has been shown to suppress the activation and proliferation of CD4+ T-cells through several mechanisms in both *in vitro* cell culture systems and *in vivo* in the mouse model of gonococcal infection. These mechanisms include Opa protein binding of CEACAM1 receptor of T-cells, induction of immunosuppressive cytokines and cell surface molecules in dendritic cells, and induction of TGF-β production through an unknown mechanism (Boulton and Gray-Owen, 2002; Liu et al., 2012; Zhu et al., 2012). These studies indicate that there are numerous mechanisms working in concert by which
*N. gonorrhoeae* evades the host adaptive immune system. These data and data demonstrating that previously infected patients are at high risk for re-infection with *N. gonorrhoeae*, including re-infection with the same serovar (Fox et al., 1999), indicate a poor humoral immune response and lack of a protective adaptive immune response against *N. gonorrhoeae*.

### 1.4.3 The role of *N. gonorrhoeae* surface molecules in the innate and adaptive immune response

*N. gonorrhoeae* is highly adapted to its human host, and multiple components of the bacterial cell wall interact directly with host receptors and serum factors. These components include gonococcal lipooligosaccharide (LOS) and bacterial proteins such as the Opa, porin, and pilin proteins.

LOS is the predominant glycolipid of the gonococcal outer membrane. Structurally, LOS is similar to lipopolysaccharide (LPS) that is found in the outer membranes of most gram-negative bacteria. Gonococcal LOS consists of the lipid A and core oligosaccharide of LPS, but lacks the O-antigen, a glycan polymer side chain attached to the core oligosaccharide (Preston et al., 1996). The carbohydrate moieties of *N. gonorrhoeae* LOS mimic carbohydrates in glycosphingolipids of human cells and is considered to be one mechanism of host immune evasion (Mandrell and Apicella, 1993). Antigenic variation of LOS composition can alter the ability of *N. gonorrhoeae* to invade host tissues, maintain serum resistance, and/or confer resistance to host antimicrobial peptides (Balthazar et al., 2011; van Putten, 1993). Variation of the carbohydrate composition of the LOS core is mediated through the action of glycosyl transferases encoded by the *lgt* genes (Yang, 1996). The lipid A of LOS in *N. gonorrhoeae* is typically decorated with phosphoethanolamine (PEA) at the 4’ position, which facilitates the interaction between outer membrane protein PorB and host complement regulatory factor C4b to
enhance serum resistance (Lewis et al., 2013). PEA decoration also promotes gonococcal resistance to host cationic antimicrobial peptides (Lewis et al., 2009). \textit{N. gonorrhoeae} which are unable to add PEA to lipid A through a deletion of PEA transferase (\textit{lptA}) have a demonstrated fitness defect in both the murine model and the male human challenge model of infection (Hobbs et al., 2013). Other modifications to the lipid A group, such as the addition of phosphoryl moieties, result in different potencies in lipid A activation of TLR4 pathways and the induction of proinflammatory cytokines (John et al., 2008; Liu et al., 2010).

The type IV pilus of \textit{N. gonorrhoeae} is a multi-protein complex that forms a protrusion from the outer membrane of the bacteria. Type IV pili are moderately conserved across gram-negative bacteria and play a role in motility, transformation, biofilm formation, and pathogenesis (Craig et al., 2004). Gonococcal pili are required for the natural competence of the bacteria and likely contribute to the bacteria’s rapid acquisition of antibiotic resistance (Seifert et al., 1990). A role of pilin in pathogenesis is their ability to increase adhesion to host cells (Rudel et al., 1992). CD46 has previously been reported as the receptor for Neisseria pili, though another study demonstrated that binding efficiencies of piliated \textit{N. gonorrhoeae} did not correlate with CD46 expression (Källström et al., 1997; Kirchner et al., 2005). Though considered to be a virulence factor, pilin does not appear to be strictly required for infection, as a \textit{N. gonorrhoeae} mutant unable to express pilin protein due to a deletion in the promoter region of \textit{pilE} was still able to establish infection in male volunteers (unpublished data discussed in Cohen and Cannon, 1999). Antigenic variation of pilin is likely a mechanism of immune system evasion. Antigenic variation results from the recombination of \textit{pilE} with one of many silent \textit{pilS} pseudogenes (Haas et al., 1992). Pilin variation has been observed in both natural and experimental gonorrhea infection (Seifert et al., 1994; Swanson, 1987).
The Opa proteins of *N. gonorrhoeae* undergo phase variable expression during infection and are another example of immune evasion through antigenic variation. In the *N. gonorrhoeae* strain FA1090, there are 11 Opa loci which express at least 8 antigenically distinct Opa proteins (Dempsey et al., 1991). While *N. gonorrhoeae* not expressing any Opa proteins are able to establish infection in the urethra of male volunteers, *N. gonorrhoeae* isolates recovered from the urethra after inoculation with an *N. gonorrhoeae* isolate with expression of all Opa genes phased off increasingly expressed various Opa proteins during the course of infection (Jerse et al., 1994). Members of the Opa family proteins of *N. gonorrhoeae* are thought to play a role in bacterial adhesion through interaction with host cell heparin sulfate proteoglycans (HSPG) (Grassmé et al., 1997). In addition to avoiding immune recognition through antigenic variation, Opa proteins are thought to modify immunologic response through binding of carcinoembryonic antigen-related family of adhesion molecules (CEACAM) (Gray-Owen et al., 1997; Virji et al., 1996a, 1996b). *N. gonorrhoeae* binding to the CEACAM1 receptor expressed on primary human CD4+ T-cells suppresses their activation and proliferation (Boulton and Gray-Owen, 2002). The Opa-CEACAM interaction on dendritic cells may affect immune responses to other co-infecting pathogens such as HIV (Yu et al., 2013). As noted in the previous section, this interaction presents another mechanism by which *N. gonorrhoeae* evades a protective adaptive host immune response.

The most abundant outer membrane protein of *N. gonorrhoeae* is the 34-36 kDa trimeric membrane protein designated PorB (Sparling, 2008). While different strains of *N. gonorrhoeae* carry antigenically distinct alleles of PorB, unlike the other predominate surface molecules mentioned above, gonococcal porin does not undergo significant antigenic variation during infection and the protein has been studied for its potential as a vaccine antigen (Massari et al.,
In addition to its role in allowing small molecule transport across the outer membrane, the porin of *N. gonorrhoeae* has several identified functions in the virulence of the bacteria. Gonococcal porin has been shown to transfer from the bacterial outer membrane to host cell membranes and then translocate to the mitochondria of immortalized human T cells and epithelial cells. Some researchers have reported that PorB translocation to the mitochondria induces host cell apoptosis, which may play a role in the epithelial shedding that occurs during infection (Muller, 2000). Treatment of primary human PMNs with isolated porin protein PorB leads to the downregulation of opsonin-dependent cell surface receptors (Bjerknes et al., 1995). Some serotypes of PorB enhance serum resistance by binding to factor H, a downregulator of the alternative complement pathway (Ram et al., 1998).

Thus, gonococcal surface molecules play multiple roles during infection pathogenesis, including resistance to host antimicrobial responses, evasion of immune mediated recognition, and manipulation of the developing immune response.

### 1.4.4 Other immune evasion mechanisms of *N. gonorrhoeae*

*N. gonorrhoeae* has evolved numerous mechanisms to avoid killing by antimicrobial responses initiated by the innate immune recognition of the bacteria. These include resistance to complement as described above, resistance to killing by host antimicrobial peptides, and killing by the PMNs that infiltrate the site of infection.

*N. gonorrhoeae* resistance to killing by PMNs is well-documented (Criss and Seifert, 2012). The gonococcal metalloprotease NG1686 and several DNA repair proteins, including RecN and RecA, have been shown to mediate resistance to external killing by PMNs and resistance to oxidative killing by hydrogen peroxide (Stohl and Seifert, 2006; Stohl et al., 2005, 2012). While the Rec proteins are important in the repair of oxidatively damaged DNA,
NG1686 appears to promote resistance to oxidative PMN killing through effects on type IV pilus production (Stohl et al., 2013). How the pilus promotes resistance to PMN antimicrobial responses is unknown. *In vitro*, PMNs have shown the capacity to kill only a subset of *N. gonorrhoeae*, as 40-70% of bacterial viability is retained in the presence of PMNs following an initial killing phase (Criss et al., 2009). The mechanisms by which they are able to survive non-oxidative host antimicrobial factors has not been well elucidated.

A partial explanation of the resistance to killing by host PMNs may be resistance to host antimicrobial peptides. Resistance to host antimicrobial peptides is mediated by several mechanisms, including LOS structure as noted above as well as via the *mtr* efflux system (Shafer et al., 1998). In the murine model of infection, *mtr* mutants of *N. gonorrhoeae* have a survival defect *in vivo* compared to *N. gonorrhoeae* with a functional *mtr* efflux system (Jerse et al., 2003). This system has also demonstrated an ability to mediate resistance to hydrophobic antimicrobial agents, including progesterone which is found at high concentrations in the female genital tract (Hagman et al., 1995).

1.5 *N. gonorrhoeae* peptidoglycan properties and interactions with the immune system

1.5.1 Peptidoglycan fragment release in *N. gonorrhoeae*

*N. gonorrhoeae*, along with *Bordetella pertussis*, is one of the few pathogenic gram-negative bacteria known to release biologically relevant quantities of peptidoglycan (PGN) monomers during growth and infection (Cookson et al., 1989; Melly et al., 1984). *N. gonorrhoeae* fail to recover around 15% of their PGN during growth, compared to 1-4% in most other gram negative bacteria (Woodhams et al., 2013). PGN monomers released by *N. gonorrhoeae* (Figure 1.2) contain one GlcNAc-1,6-anhydro-MurNAc disaccharide unit linked to a peptide, 80% of which are the L-alanine-D-glutamic acid-*meso*-diaminopimelic acid tripeptide,
with the remaining 20% composed mostly of terminal D-alanine containing tetrapeptide (Sinha and Rosenthal, 1980). In *Bordetella pertussis*, the bacteria release tetrapeptide-containing peptidoglycan monomers that are known as tracheal cytotoxin (TCT) due to their ability to damage ciliated epithelial cells (Cookson et al., 1989; Goldman et al., 1982). Monomeric PGN fragments from *N. gonorrhoeae* were later shown to damage the mucosa of human fallopian tubes (Chan et al., 2012; Melly et al., 1984). Purified TCT has been shown to induce expression of IL-1β and IL-6 in human monocytes and likely contributes to inflammation during *B. pertussis* infection (Dokter et al., 1994). *N. gonorrhoeae* PGN has arthropathic properties when injected into rats and probably contributes to symptoms of disseminated gonococcal infection (Fleming et al., 1986). The interaction of released PGN with the immune system that trigger these inflammatory events and the role the PGN release by *N. gonorrhoeae* plays in pathogenesis *in vivo* has not been well studied. There are numerous proteins involved in the remodeling of the cell wall during growth, and recently several proteins have been implicated in the production and release of monomeric PGN in *N. gonorrhoeae*.

### 1.5.2 Peptidoglycan recycling in *N. gonorrhoeae*

The cell wall of *N. gonorrhoeae* is comprised of polymeric PGN consisting of long chains of alternating sugars, N-acetylglucosamine and N-acetylmuramic acid. Attached to the N-acetylmuramic acid is a peptide chain 3-5 amino acids in length. In *N. gonorrhoeae* and other gram-negative bacteria the peptide consists of L-alanine, D-glutamic acid, *meso*-diaminopimelic acid (DAP), and D-alanine. There are several classes of enzymes involved in the remodeling of the cell wall during growth. A model of PGN recycling in *N. gonorrhoeae* as described in the following text is depicted in Figure 1.3 (Adapted from Garcia and Dillard, 2008).
Amidases are responsible for cleaving the bond between the L-alanine and the N-acetylmuramic acid and generating fragments for PGN recycling during growth. In *N. gonorrhoeae* the periplasmic amidase AmiC cleaves this bond, freeing N-acetylmuramic acid-β-1,4-N-acetylglucosamine disaccharide from the sacculus. AmiC is necessary for daughter cell separation during division and also functions as one of several *N. gonorrhoeae* autolysins (Garcia and Dillard, 2006). AmpD is a second characterized *N. gonorrhoeae* amidase that cleaves the N-acetylmuramyl-L-alanine bond of cytoplasmic PGN fragments. Mutation of *ampD* results in a buildup of PGN in the cytoplasm, an increased metabolism of PGN fragments, and a reduced release of extracellular PGN fragments (Garcia and Dillard, 2008).

Endopeptidases and carboxypeptidases cleave the bonds between amino acids on the peptide chain, including cross-linkages. NG1686 is a periplasmic metallopeptidase with both endopeptidase and carboxypeptidase activity. Deletion of *ng1686* results in altered colony morphology, but not bacterial cell size or cell shape (Stohl et al., 2012). The precise role of NG1686 in PGN recycling and cell wall rebuilding is not well described. NG1686 also has separate roles in *N. gonorrhoeae* pathogenesis, including resistance to H₂O₂ and PMN-mediated killing and type IV pilus formation as mentioned in Section 1.4.4 (Stohl et al., 2012, 2013).

Other proteins with endopeptidase and carboxypeptidase activities are penicillin binding protein (PBP) 3 and 4. Deletion of either PBP in *N. gonorrhoeae* is tolerated, but deletion of both PBP3 and PBP4 results in a slowed growth phenotype and altered cell morphology (Stefanova et al., 2003). LdcA is a fourth known carboxypeptidase. LdcA is a cytoplasmic protein responsible for removing the terminal D-alanine on the tetrapeptide chain from PGN monomers.

An additional critical component of PGN recycling in *N. gonorrhoeae* is the permease AmpG, which mediates the uptake of periplasmic PGN fragments and their transport into the
cytoplasm. Deletion mutants for ampG do not have detectable levels of PGN in the cytoplasm, and the release of PGN into the culture supernatant by these mutants is increased seven-fold (Garcia and Dillard, 2008).

Additional proteins found within the cytoplasm that further degrade PGN fragments provided by AmpG include the N-acetylglucosaminidase NagZ, which produces free N-acetylglucosamine, and AmhK, which eliminates the 1,6-anhydro bond by phosphorylation of the N-acetylmuramic acid. These proteins are well-described in E. coli and gene homologues have been found in N. gonorrhoeae FA1090 whole genome sequencing data (Garcia and Dillard, 2008). Mutations in these genes to test the role of their protein products in growth, cell size and shape, colony morphology, and PGN release have not been studied.

Finally, lytic transglycosylases cleave the N-acetylmuramic acid-β-1,4-N-acetylglucosamine bond, creating a 1,6-anhydro bond on the muramic acid sugar. Most N. gonorrhoeae express seven lytic transglycosylases. Two of the seven lytic transglycosylase proteins are encoded on the 57-kb gonococcal genetic island (GGI), which is expressed by approximately 80% of all N. gonorrhoeae (Ramsey et al., 2011). All forms of the GGI encode the lytic transglycosylase LtgX. N. gonorrhoeae with ltgX deleted does not secrete detectable levels of DNA during growth, suggesting that LtgX is critical for type IV secretion (Kohler et al., 2007). 79% of GGI forms encode a second lytic transglycosylase, AtlA (Dillard and Seifert, 2001). A recombinantly expressed AtlA fusion protein demonstrated the ability to digest gonococcal PGN in vitro, confirming its activity as a lytic transglycosylase (Kohler et al., 2007). In vivo, a N. gonorrhoeae mutant which contained a point mutation in the putative active site of atlA showed a 500-fold reduction in DNA donation (Chan et al., 2012). Taken together, these data show that one
function of some lytic transglycosylases is to produce breaches in the PGN cell wall required for
the function of type IV secretion machinery.

In *N. gonorrhoeae*, the lytic transglycosylase LtgC is important for normal bacterial growth;
mutation of *ltgC* was shown to affect gonococcal growth, producing smaller colonies on agar and
appearing to have a slower growth rate in liquid culture as compared to the isogenic wild type *N.
gonorrhoeae*. Although the *ltgC* mutant lacks a lytic transglycosylase, it undergoes autolysis
more readily than its isogenic parental strain. Finally, electron micrograph analysis revealed that
*ltgC* mutants failed to fully separate, suggesting that LtgC acts on PGN specifically at the septum
of the dividing cell to promote separation (Cloud and Dillard, 2004).

LtgB and LtgE are currently of unknown function. Deletion of *ltgB* in *N. gonorrhoeae* does
not affect PGN fragment release or growth. Interestingly, expression of LtgB in *E. coli* was able
to substitute for the function of lambda endolysin and cause cell lysis, indicating that the protein
does have lytic transglycosylase activity (Kohler, 2005). A point mutation of *ltgE* also did not
affect PGN monomer release, though PGN multimer release appeared slightly elevated (Cloud-
Hansen et al., 2008).

The two remaining gonococcal lytic transglycosylases, LtgA and LtgD, have been well
studied and have shown to be responsible for producing the 1,6-anhydro PGN monomers
released by *N. gonorrhoeae*. LtgA is predicted to be a lipoprotein 616 amino acids long and with
a molecular weight of 67.5kDa. A deletion mutant of *ltgA* in *N. gonorrhoeae* has a 40% 
reduction in the release of PGN monomers compared to wild type (Chan et al., 2012). LtgD is
also a predicted lipoprotein, and is 363 amino acids in length with a molecular weight of
38.5kDa. A deletion mutant of *ltgD* showed a 60% reduction in the release of PGN monomers
(Chan et al., 2012). These data suggest non-redundant functions for each of these lytic
transglycosylases in digesting PGN and producing PGN monomers. The LtgA and LtgD proteins may have different spatial and temporal expression, or they may be responsible for digesting PGN fragments of different sizes. Elimination of PGN monomer release in *N. gonorrhoeae* can be achieved by deleting both *ltgA* and *ltgD*. *N. gonorrhoeae* lacking both LtgA and LtgD do not release PGN monomers, but rather PGN multimers that consist of dimers, trimers, and other larger fragments. Though *N. gonorrhoeae* lacking these enzymes fail to release recyclable 1,6-anhydromuropeptides, the bacteria do not have a growth defect in culture (Cloud-Hansen et al., 2008). It remains unclear what selective pressures are present in the host environment to cause *N. gonorrhoeae* to maintain LtgA and LtgD.

1.5.3 Other peptidoglycan modifications

In addition to the proteins involved in the breakdown and re-building of the PGN of the cell wall, there are proteins that function to make specific modifications to PGN. Many bacteria, including *N. gonorrhoeae*, O-acetylate their PGN (Blundell et al., 1980). The O-acetylation of PGN occurs at the C6 carbon of the muramyl residues by the action of an O-acetyltransferase. In *N. gonorrhoeae* this occurs through a recently identified O-acetyltransferase, PatB (also referred to as Ape2), in conjunction with an unknown acetate transport protein (Moynihan and Clarke, 2010). *N. gonorrhoeae* O-acetylate approximately 34-52% of their PGN (Swim et al., 1983). PGN modification via O-acetylation is known to increase PGN resistance to lysozyme digestion in many pathogenic bacteria, including *N. gonorrhoeae* (Bera et al., 2006; Dillard and Hackett, 2005; Moynihan and Clarke, 2011; Rosenthal et al., 1982, 1983). The PGN O-acetyltransferase in *Staphylococcus aureus* can modulate host cytokine responses to the bacteria by blocking lysozyme digestion and subsequently suppressing inflammasome activation. (Shimada et al.,
The precise role that O-acetylation of *N. gonorrhoeae* PGN plays in the immune response to infection with the bacteria is unknown.

A second PGN modification that has been demonstrated in gram-positive bacteria and, more recently, in the gram-negative bacteria *Helicobacter pylori*, is N-deacetylation of the PGN sugars (Boneca et al., 2007; Wang et al., 2009). Like, O-acetylation of PGN, N-deacetylation confers resistance to digestion by host lysozyme and suppress host inflammatory immune responses (Wang et al., 2010). No N-deacetylation enzymes have been identified in *N. gonorrhoeae* and thus a role of N-deacetylation in the pathogenesis of infection has not been established.

### 1.5.4 Immune system recognition of peptidoglycan

PGN is a microbe-associated molecular pattern important in the immune recognition of both pathogenic and commensal bacteria. PGN is recognized by several innate immune receptors, including the NOD1, NOD2, and the PGRPs.

In humans, there are four PGRPs, which bind both extracellular and intracellular PGN (Liu et al., 2000, 2001; Lu et al., 2005). In mammals, they are designated PGLYRP1, PGLYRP2, PGLYRP3, and PGLYRP4. While all PGLYRPs have bactericidal activity, PGLYRP2 uniquely has amidase activity that contributes to its antimicrobial activity (Gelius et al., 2003; Wang et al., 2003). PGLYRP1 is primarily expressed in the granules of neutrophils and eosinophils, contributing to their anti-bacterial activity (Cho, 2005). PGLYRP2 is expressed primarily in the liver, where it is then secreted into the blood, but expression also be induced in epithelial cells, namely the skin and gastrointestinal tract, through the action of cytokines such as IL-1β and TNF-α (Royet et al., 2011; Zhang et al., 2005). Finally, PGLYRP3 and PGLYRP4 are expressed in the skin and mucous membranes (Royet et al., 2011). Some insect PGRPs have demonstrated specificity for DAP-type or Lys-type PGN (Leulier et al., 2003), and the mammalian PGLYRP1
preferentially binds multimeric PGN (Liu et al., 2000). All four PGRPs have been implicated in protection against colitis, as mice deficient in any of the four PGRPs have enhanced susceptibility to dextran sulfate sodium-induced colitis compared to wild type mice (Saha et al., 2010). The likely mechanism for this protection is through innate immune regulation of gut commensal bacteria.

The second set of receptors that detect bacterial peptidoglycan are the including the nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and 2 (NOD2) proteins. Activation of NOD1 and NOD2 by PGN components D-glutamyl-meso-diaminopimelic acid (iE-DAP) or muramyl dipeptide (MDP), respectively, leads to the induction of NF-κB and subsequent inflammatory cytokine production (Chamaillard et al., 2003; Girardin et al., 2003a, 2003b; Inohara, 2003). Due to the requirement of the meso-DAP moiety, which is not present in the PGN of gram-positive bacteria, NOD1 is primarily a sensor of gram-negative bacteria. In contrast, NOD2 is a general sensor of all bacteria. High levels of NOD2 expression is restricted to phagocytic cells and some specialized epithelial cells, like Paneth cells of the ileal crypts, while NOD1 is more ubiquitously expressed (Inohara et al., 1999; Ogura et al., 2003). The ligand specificity and tissue distribution differences may indicate distinct functions of these receptors in bacterial sensing in the host despite similar downstream signaling consequences. NOD1 has been shown to play an important role in the host response to Helicobacter pylori (Viala et al., 2004). Mutations in NOD2 are associated with development of familial Crohn’s disease, an inflammatory bowel disease. Mutant variants of NOD2 have defective tolerogenic responses to commensal flora in the intestine (Kullberg et al., 2008; Petnicki-Ocwieja et al., 2009). One mechanism for the defective tolerogenic responses may be due to the NOD2 mutant’s inability to recruit ATG16L1, a protein involved in the autophagic functions of NOD1
and NOD2 (Travassos et al., 2009). Activation of NOD2 by MDP has been shown to stimulate production of antimicrobial peptides including HNP1 and \( \beta \)-defensin-2 (Voss et al., 2006; Yamamoto-Furusho et al., 2010). NOD2 signaling also plays several roles in coordinating the development of adaptive immune responses. For example, NOD2 is known to mediate MHC class II antigen-specific CD4+ responses and facilitate polarization of Th2 cells in response to those antigens (Cooney et al., 2009; Magalhaes et al., 2008). Recent studies have characterized NOD activation in response to \( N.\ gonorrhoeae \) exposure. In HEK293 reporter cell lines, \( N.\ gonorrhoeae \) culture supernatants were shown to be potent activators of NOD1, but poorly activating of NOD2 (Mavrogiorgos et al., 2013). Our studies present here provide a mechanism for this differential activation, as \( N.\ gonorrhoeae \) release large quantities of PGN monomers that activate NOD1 but not NOD2. The role that NOD1 and NOD2 signaling has in \( N.\ gonorrhoeae \) infection pathogenesis has not been studied.

The role of TLR2 in peptidoglycan recognition has not been resolved, as conflicting reports exist in the literature regarding the subject. TLR2 was initially identified as a sensor of PGN in \( TLR2^{-/-} \) mouse macrophages and in fibroblasts transfected with human TLR2 using crude preparations of PGN from \( Staphylococcus \) species (Takeuchi et al., 1999b; Yoshimura et al., 1999). However, a report by Travassos et al. demonstrated that highly purified preparations of PGN from multiple gram-positive and gram-negative bacterial species were unable to activate TLR2 (Travassos et al., 2004). The purification process involved boiling with SDS to inactivate autolysins and remove non-covalently bonded proteins and LPS. Crude PGN preps were then treated with \( \alpha \)-amylase and trypsin to remove glycogen and covalently bound proteins, respectively, followed by a hydrofluoric acid wash of gram-positive PGN to remove secondary polysaccharides (such as teichoic acid) covalently bound to the PGN. Final treatment gram-
positive and gram-negative PGN involved 8M LiCl and 0.1M EDTA to remove any polypeptidic contamination and finally an acetone wash to remove lipoteichoic acids or any traces of LPS (Girardin et al., 2003a). A subsequent paper replicated the purification methods used by Travassos et al. using S. aureus PGN and showed that the highly purified PGN could activate TLR2 (Dziarski and Gupta, 2005). Some studies show that TLR2 recognizes PGN-associated lipoproteins, while another study demonstrates that TLR2 of mouse macrophages recognize multimeric PGN from Δlgt S. aureus, which lack lipidded prelipoproteins. (Müller-Anstett et al., 2010; Travassos et al., 2004). A contradicting study published around the same time showed the polymeric PGN prepared from Δlgt S. aureus did not activate human TLR in a reporter cell line (Volz et al., 2010). There is evidence that TLR2 co-localizes with NOD2, possibly confounding the mechanism by which recognition occurs in in vitro systems (Müller-Anstett et al., 2010; Watanabe et al., 2004). Additional studies are needed to understand the role, if any, TLR2 plays in PGN recognition and PGN recognition pathways involving NOD1 and NOD2.

1.6 The murine model of N. gonorrhoeae infection

There are a limited number of well-characterized models available to study N. gonorrhoeae infection in vivo. While an experimental human challenge model of infection has been established, several factors limit the extent of its use for immunologic studies: 1) infection is limited to men, 2) studies are expensive and involve a low number of subjects, and 3) ethical considerations require rapid antibiotic treatment following the onset of infection (Cohen and Cannon, 1999). Thus, many studies of N. gonorrhoeae infection in vivo rely on an established murine model of infection.

Initial attempts at stable genital colonization of female mice failed, with very few mice having recoverable amounts of N. gonorrhoeae following inoculation and N. gonorrhoeae was
only recoverable for several days (Johnson et al., 1989; Streeter and Corbeil, 1981). The presence of vaginal flora and vaginal PMNs negatively affect the ability of *N. gonorrhoeae* to colonize the mouse vagina and consequently inoculation during proestrus stage of the estrous cycle was more favorable to colonization (Braude, 1982). In 1990, Taylor-Robinson et al. published a report describing the ability of 17β-estradiol-treated germ-free BALB/c mice to maintain long-term vaginal colonization with *N. gonorrhoeae* (Taylor-Robinson et al., 1990). These observations lead to the development of a murine model of infection where long-term vaginal colonization with *N. gonorrhoeae* could be achieved with 17β-estradiol treatment and regular antibiotic administration to suppress the overgrowth of commensal flora (Jerse, 1999). The exact mechanisms by which estradiol promotes susceptibility of mice in colonization with *N. gonorrhoeae* is unknown, but suppressing the influx of PMNs and dampening the inflammatory response is thought to play a role (Ralston et al., 2009). In mice treated with three subcutaneous injections of a water-soluble 17β-estradiol spaced 48 hours apart, *N. gonorrhoeae* can be recovered from the genital tract for an average of 10 days (Song et al., 2008). Colonization time can be extended through the use of slow-release estradiol pellets or additional subcutaneous injections (Jerse, 1999).

Access to clean animal housing facilities is critical for utilizing the murine model of *N. gonorrhoeae* infection. Colonization of the mucosal surfaces with commensal bacteria can persist despite a combined oral and intraperitoneal antibiotic regimen. The presence of enteric gram-negative rods and catalase-positive, gram-positive cocci is negatively associated with persistence of gonococcal infection in mice (Jerse, 1999; Streeter and Corbeil, 1981). In my own studies, a common commensal contaminate, *Staphylococcus xylosus*, did not impact the establishment of vaginal infection with *N. gonorrhoeae*, but was associated with accelerated
clearance of *N. gonorrhoeae* (Figure 1.4). The precise mechanisms that promote accelerated clearance of *N. gonorrhoeae* in the presence of these microorganisms are currently unknown. Conversely, the presence of lactobacilli has been correlated with an increase in bacterial recovery from mouse vaginal swabs and has been shown to enhance *N. gonorrhoeae* growth on agar (Jerse, 2002; Jerse et al., 2011). In our studies, mice that test positive for these commensals are removed from the study and not considered during data analysis to ensure that data are not compromised by variability in commensal colonization. However, *N. gonorrhoeae* interaction with commensals may be an important area of study, in particular when considering genital infection in women and the persistence of infection despite the presence of a diverse vaginal microbiota (Newton et al., 2001).

The choice of mouse strain is another important consideration, as several mouse strain-dependent differences in susceptibility and immune response to *N. gonorrhoeae* have been observed (Packiam et al., 2010; Streeter and Corbeil, 1981). For example, while vaginal infection in 17β-estradiol treated BALB/c mice typically results in the influx of PMNs, this does not occur during infection with C57BL/6 mice (Packiam et al., 2010). C3H/HeN mice are resistant to infection with *N. gonorrhoeae*, and this resistance is not mediated by an increased inflammatory response but by a currently undefined mechanism (Packiam et al., 2010). Prior to the establishment of the murine model of *N. gonorrhoeae* infection, C3H/HeJ mice were found to be more resistant to vaginal infection with *N. gonorrhoeae* than C3H/HeN mice. The authors concluded the observed resistance of the C3H/HeJ to *N. gonorrhoeae* colonization was due to both high numbers of PMNs in the vaginal mucus and high numbers of recovered gram-negative flora (Streeter and Corbeil, 1981). These data would later be critical in understanding why *N.*
*N. gonorrhoeae* is a poor colonizer of the mouse genital tract and informing the protocol for the development of an effective mouse model.

In addition to the characteristic PMN influx during infection, the murine genital tract infection model of *N. gonorrhoeae* infection shares several other characteristics with those observed in infection in humans. Despite an ability to clear *N. gonorrhoeae* infection, mice do not develop a robust humoral immune response and are susceptible to re-infection (Song et al., 2008). Cytokines such as IL-6, TNF-α, and IL-17 that have been found to be elevated during infection in humans as detailed in section 1.4.2 have also been found to be elevated in mice (Feinen et al., 2010; Jerse et al., 2011; Packiam et al., 2010). Surprisingly, a positive selection for Opa-expressing *N. gonorrhoeae* has also been observed in the mouse model of infection, despite the known specificity of Opa for human CEACAM and the lack of a known receptor in mice (Cole et al., 2010). In contrast, there is no known receptor for pilin in mice, and a loss of piliation in isolates recovered from mice suggests that piliation does not play a role in the murine model of infection (Jerse, 1999).

There are clear limitations to use of a murine model of infection, including differences in physiology, host cell surface receptors, and immune signaling pathways. For example, mice express three additional TLRs, one of which (TLR13) has demonstrated a role in sensing of bacterial rRNA (Oldenburg et al., 2012). Mouse NOD1 is able to recognize TCT while human NOD1 cannot; ligand differences of NOD2 signaling in mice and humans have not been well studied (Magalhaes et al., 2005). Other differences between mouse and human immune responses include distribution of immune cell subsets, expression of immune receptors, expression of Ig isotypes, and activation of immune cell types (Mestas and Hughes, 2004). These
should be taken into consideration when conducting immunological studies in mice, perhaps especially in consideration of *N. gonorrhoeae*, which does not naturally infect mice.

Specific to the vaginal mouse model of infection, there are some physiological differences. This includes differences in vaginal pH, with mouse vaginal pH being high than human vaginal pH, though similar to human cervical pH, where *N. gonorrhoeae* can establish infection (Muench et al., 2009; Singer, 1975). There is also notably no period of menstrual bleeding in mice, and the menstrual cycle in women has been thought to play a role in gonococcal pathogenesis, including dissemination of infection (O’Brien et al., 1983). Finally, as mentioned previously, the microflora of the mouse vaginal tract are different than that of women, though this consideration must be taken in the context of the infection model, where antibiotics are used to reduce the population of commensal species. The ability of *N. gonorrhoeae* to access certain critical nutrients in the mouse genital tract, such as iron, is unknown; iron sources such as transferrin (Tf) and lactoferrin (Lf) are unavailable in mice because the gonococcal Tf and Lf receptors are specific to the human ligand (Jerse et al., 2011).

Thus, careful considerations of the limitations of using a mouse model of *N. gonorrhoeae* must be taken in evaluating experimental results. The murine model of infection, despite the differences mentioned, does mimic many characteristics of infection observed in humans. The model provides a starting point for immunological studies that include vaccine development, and can contribute mechanistic data through the use of knockout mice. An increase in the availability of transgenic mice expressing human pilin and Opa receptors would greatly enhance the relevancy of this model in studying *N. gonorrhoeae* infection and potential vaccine strategies.
1.7 Vaccination against *N. gonorrhoeae*

The development of a vaccine against *N. gonorrhoeae* has been unsuccessful. Antigenic variation of outer membrane proteins and active suppression of the protective adaptive immune response, as described in section 1.4, are attributed to these failures. Still, in a limited number of cases, serovar-specific acquired immunity involving the gonoccocal outer membrane protein PorB has been reported (Buchanan et al., 1980). An infection experiment conducted in chimpanzees showed the development of short-term resistance to re-infection (Kraus et al., 1975). Due to the decline in the use of chimpanzees as experimental models for both ethical and financial reasons, these experiments have not been repeated to confirm the result or elucidate the mechanisms by which resistance to re-infection occurred. These cases all represent strain-specific immunity, a challenge that will need to be overcome to achieve development of a broadly acting vaccine.

To date, two *N. gonorrhoeae* vaccine candidates have entered clinical trials. An early trial involved a crude whole-cell vaccine formulation. The vaccine was well tolerated and was able to produce antibodies in 90% of the vaccinated group, but the vaccine ultimately failed to protect against infection (Greenberg, 1975; Greenberg et al., 1974). The second vaccine attempt was a large clinical trial in Korea and utilized purified *N. gonorrhoeae* pilin of a single pilus type. The efficacy of this vaccine in women was unable to be assessed, as no women enrolled in the study contracted gonorrhea. There was no protective effect in men, as about equal numbers of men who received the vaccine contracted gonorrhea as those who received the placebo (Boslego et al., 1991). Despite the poor efficacy outcome, the vaccine did induce anti-gonococcal pilin antibodies, including cross-reactive antibodies to pilin from a heterologous strain (Boslego et al., 1991).
Recent insights into the type of adaptive immune response important in the development of protective immunity to *N. gonorrhoeae* have been elucidated in the murine model of infection. A report by Liu et al. demonstrated that *N. gonorrhoeae* were able to selectively suppress the development of Th1 and Th2 cells through a TGF-β-dependent mechanism. Importantly, the study demonstrated that blockade of the TGF-β signaling pathway using an anti-TGF-β antibody was able to redirect the Th response to a Th1/Th2 response *in vitro* (Liu et al., 2012). Mice treated with anti-TGF-β antibody during infection with *N. gonorrhoeae* demonstrated accelerated clearance of the bacteria, development of anti-gonococcal antibodies, and resistance to reinfection (Liu and Russell, 2011). The use of knockout strains deficient in either Th1 or Th2 responses showed that the accelerated clearance of the bacteria was due to the Th1 response, but resistance to re-infection was dependent on both Th1 and Th2 responses (Liu and Russell, 2011). Thus the induction of Th1 and possibly Th2 responses may be a critical component of a vaccine. Local administration of microencapsulated IL-12, a proinflammatory cytokine known to activate Th1 cells (Macatonia et al., 1995), during *N. gonorrhoeae* infection in mice caused accelerated clearance of the bacteria as well as resistance to re-infection (Liu et al., 2013). If similar responses can be achieved in humans, local administration of proinflammatory cytokines to induce a specific Th cell response may be a novel therapeutic approach to treatment of *N. gonorrhoeae* infection, particularly considering the high rate of re-infection in the population (Brooks et al., 1978; Hosenfeld et al., 2009).

The development of a *N. gonorrhoeae* vaccine is becoming more critical as antibiotic resistance becomes wide-spread and the risk of incurable gonorrhea rises. More research is needed to identify antigens and stimulatory molecules that will enhance Th1 and Th2 activation. Important to the development of a vaccine are further studies on immune system interaction
between *N. gonorrhoeae* and the host during infection, including mechanisms that mediate evasion of a protective adaptive immune response and the induction of inflammatory responses that likely contribute to pathogenesis and transmission. The studies presented here report on the roles of *N. gonorrhoeae* PGN on cytokine production and immune receptor activation, as well as the role of lytic transglycosylases LtgA and LtgD *in vivo*. We provide a framework for further studies on *N. gonorrhoeae* PGN and the activation of NOD1 and NOD2 in *N. gonorrhoeae* pathogenesis.
Figure 1.1. Current understanding of the immune response to \textit{N. gonorrhoeae} infection.
Upon recognition of \textit{N. gonorrhoeae} by antigen presenting cells, depicted here as a dendritic cell (DC), subsequent cytokine signaling results in the polarization of T-cells to Th17, which produce cytokines such as IL-17 and IL-22. In response, epithelial cells produce chemokines in the CXC family, which results in the influx of polymorphonuclear leukocytes (PMNs). \textit{N. gonorrhoeae} is resistant to PMN killing through a combination of resistance to phagocytosis and resistance to both oxidative and non-oxidative antimicrobial factors in cases when the bacteria are internalized. Suppression of Th1/Th2 polarization occurs through a TGF-β-dependent mechanism. \textit{N. gonorrhoeae} can also inhibit the polarization and proliferation of CD4+ T-cells through direct interaction with surface CEACAM1 molecules or indirectly through DCs (not shown). This model represents data obtained primarily through \textit{in vivo} studies using the murine model of infection and \textit{in vitro} studies using immortalized and primary human and mouse cells. Cytokines IL-6, IL-17, and IL-23 have been observed to increase locally during \textit{N. gonorrhoeae} infection in men.
Figure 1.2. Structure of the peptidoglycan monomer released by *N. gonorrhoeae*.

During growth, *N. gonorrhoeae* release peptidoglycan monomers comprised of one N-acetyl-glucosamine (red) linked to one N-acetyl-muramic acid containing a 1,6 anhydro bond (blue). Attached to the N-acetyl-muramic acid is a peptide chain consisting of L-alanine, D-glutamic acid, *meso*-diaminopimelic acid and a terminal D-alanine. 80% of PGN monomers released by *N. gonorrhoeae* contain only the tripeptide; the remaining 20% are primarily tetrapeptide monomers.
Figure 1.3. Peptidoglycan recycling in *N. gonorrhoeae.*
PGN fragments are liberated from the sacculus through the action of lytic transglycosylases LtgA, LtgD, and amidase AmiC. Both PGN monomers produced by LtgA and LtgD as well as disaccharide fragments are released into the extracellular space by an unknown mechanism. PGN fragments that are not released are brought into the cytoplasm through the action of the AmpG permease. Once in the cytoplasm, PGN fragments are broken down for recycling. LdcA is a carboxypeptidase that cleaves the terminal D-alanine from the peptide chain. AmpD is another known amidase of *N. gonorrhoeae* and cleaves the peptide chain from the N-acetylglucosamine-N-acetylmuramic acid disaccharide. NagZ cleaves the bond between these two sugars. Finally, AmhK phosphorylates the 1,6-anhydromuramic acid sugar.
Figure 1.4. Persistence of *N. gonorrhoeae* is significantly reduced in mice colonized with commensal species *Staphylococcus xylosus*.

The presence of a gram-positive cocci, *Staphylococcus xylosus*, in the vaginal tract of mice causes accelerated clearance of *N. gonorrhoeae*. Mice were considered positive for *S. xylosus* (+ *S. xylosus*) if the organism was recovered from mouse vaginal swabs streaked onto HIA agar for at least 8 of the 10 days of the infection study (n = 10). Mice were considered negative for *S. xylosus* (- *S. xylosus*) if vaginal swabs indicated less than two days of positive culture (n = 11). Median infection persistence of *N. gonorrhoeae* was 10 days and 2.5 days for – *S. xylosus* and + *S. xylosus*, respectively. *N. gonorrhoeae* strain FA1090 was used in these studies and data shown are combined from two separate experiments. Data was analyzed by GraphPad Prism using a Log-Rank Test, * = P < 0.0001
Chapter 2

The role of *N. gonorrhoeae* peptidoglycan in innate immune signaling

2.1 Overview

*Neisseria gonorrhoeae* releases anhydro peptidoglycan monomers during growth through the action of two lytic transglycosylases encoded in the *N. gonorrhoeae* genome, LtgA and LtgD. Because peptidoglycan and peptidoglycan components activate innate immune signaling, we hypothesized that the activity of LtgA and LtgD would influence the host responses to gonococcal infection. *N. gonorrhoeae* lacking LtgA and LtgD caused increased host production of inflammatory cytokines IL-1β and TNF-α. Culture supernatants from ΔltgA/ΔltgD *N. gonorrhoeae*, which contain multimeric peptidoglycan fragments rather than monomers, were potent activators of host NOD2 signaling when compared to supernatants from the isogenic parental *N. gonorrhoeae* strain. Purified peptidoglycan monomers containing anhydro muramic acid produced by LtgA were poor stimulators of NOD2 while peptidoglycan monomers containing reducing muramic acid produced by host lysozyme were potent stimulators of NOD2. These data indicate that LtgA and LtgD allow *N. gonorrhoeae* to evade detection by host NOD2.

2.2 Introduction

*Neisseria gonorrhoeae* is an obligate human pathogen responsible for causing the sexually transmitted disease gonorrhea. During infection, *N. gonorrhoeae* triggers localized inflammation characterized by the influx of neutrophils. Phagocyte antimicrobial responses and host production of antimicrobial agents are initiated following recognition of bacterial
components, including LOS, lipoproteins, bacterial DNA, and peptidoglycan (Takeuchi and Akira, 2010). The cell wall of N. gonorrhoeae is comprised of polymeric peptidoglycan (PGN) consisting of long chains of alternating sugars, N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). Attached to the MurNAc is a peptide chain 3-5 amino acids in length. In N. gonorrhoeae and other gram-negative bacteria the peptide consists of L-alanine, D-glutamic acid, meso-diaminopimelic acid (DAP), and D-alanine. Amidases, endopeptidases, and lytic transglycosylases act on PGN to mediate bacterial cell wall remodeling during growth and replication.

Though cell wall remodeling typically leads to the release of some PGN-derived products, these are usually efficiently reutilized by the bacteria. Unlike most pathogenic gram-negative bacteria, N. gonorrhoeae and Bordetella pertussis are known to release intact PGN monomers in excess of the capacity of the bacteria to reclaim the material and in sufficient quantities to induce significant inflammatory responses in host tissues (Goldman et al., 1982). PGN monomers released by N. gonorrhoeae contain one GlcNAc-1,6-anhydro-MurNAc disaccharide unit linked to the L-alanine-D-glutamic acid-meso-diaminopimelic acid tripeptide (80%) and tetrapeptide bearing an additional terminal D-alanine (20%) (Sinha and Rosenthal, 1980). The tetrapeptide PGN monomers, also known as tracheal cytotoxin (TCT), were first isolated from B. pertussis as the causative agent of ciliated cell death in host airways (Goldman et al., 1982). Monomeric PGN fragments from N. gonorrhoeae were later shown to damage the mucosa of human fallopian tubes (Melly et al., 1984). The N. gonorrhoeae genome encodes seven lytic transglycosylases capable of liberating PGN from N. gonorrhoeae sacculi, but only LtgA and LtgD are responsible for production of 1,6-anhydro-MurNAc-containing PGN monomer released by N. gonorrhoeae during growth in culture (Cloud-Hansen et al., 2008).
Instead of releasing monomeric 1,6-anhydro-MurNAc-containing PGN monomers, *N. gonorrhoeae* lacking both *ltgA* and *ltgD* release a variety of multimeric PGN fragments (Cloud-Hansen et al., 2008). The role PGN monomer production by LtgA and LtgD plays in the host response to *N. gonorrhoeae* infection has not been elucidated.

PGN acts as a microbe associated molecular pattern (MAMP) that is recognized by the innate immune system in response to bacterial infections and commensal species. PGN components are recognized by several host receptors, including the nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and 2 (NOD2) proteins. Activation of NOD1 and NOD2 by PGN components D-glutamyl-meso-diaminopimelic acid (iE-DAP) or muramyl dipeptide (MDP), respectively, leads to the induction of NF-κB and subsequent inflammatory cytokine production (Chamaillard et al., 2003; Girardin et al., 2003a, 2003b; Inohara, 2003). NOD2 has been implicated in Crohn’s disease and mutations in the protein have defective tolerogenic responses to commensal flora in the intestine (Kullberg et al., 2008). Recently, *N. gonorrhoeae* culture supernatant, which contains secreted 1,6-anhydro-MurNAc-containing PGN monomers, was shown to more robustly activate NOD1, while the PGN polymer-containing lysates were found to stimulate both NOD1 and NOD2 equally (Mavrogiorgos et al., 2013). In addition to being recognized intracellularly by NOD1 and NOD2, extracellular multimeric PGN from gram-negative bacteria activates host Toll-like receptor 2 (TLR2), though some studies now suggest that TLR2 recognizes PGN-associated lipoproteins rather than multimeric PGN itself (Travassos et al., 2004). Recognition of bacterial PGN via these systems allows the host to initiate antimicrobial responses (Gold et al., 1985; Greenblatt et al., 1978).
In this study, we sought to assess the role of *N. gonorrhoeae* LtgA and LtgD-mediated PGN monomer release on the innate immune responses as well as the specific immune receptor responses to *N. gonorrhoeae* PGN monomers. Here we showed that *N. gonorrhoeae* lacking LtgA and LtgD induced production of significantly more inflammatory cytokines and greater activation of host TLR2 and NOD2 signaling when compared to wild type *N. gonorrhoeae*. The difference in NOD2 signaling was due to the inability of host NOD2 to recognize 1,6-anhydro-MurNAc-containing PGN monomers produced by the LtgA and LtgD proteins. In contrast, PGN monomers produced by the action of host lysozyme on PGN multimers lack the 1,6-anhydro bond and were potent activators of NOD2. Together, these data show that the LtgA and LtgD suppress host NOD2-mediated inflammatory cytokine signaling by converting multimeric PGN fragments generated during bacterial growth to 1,6-anhydro-MurNAc-containing PGN monomers.

### 2.3 Materials and Methods

#### 2.3.1 Generation of *N. gonorrhoeae* ltgA/ltgD mutant

*N. gonorrhoeae* strain FA1090 ΔltgA/ΔltgD was generated as described previously (Cloud and Dillard, 2002; Cloud-Hansen et al., 2008). Whole genome sequencing of FA1090 ΔltgA/ΔltgD confirmed that the entire coding region of *ltgD* was deleted while the *ltgA* gene was disrupted by deletion of the last 1420 bp of the 1850 bp *ltgA* coding region and insertion of the *ermC* gene conferring erythromycin resistance. A second construction of the ΔltgA/ΔltgD mutant, FA1090 ΔltgA/ΔltgD (KK) is described in Appendix 1.

#### 2.3.2 Generation of *N. gonorrhoeae* culture supernatants

*N. gonorrhoeae* strains were grown overnight on GCB agar plates. The bacteria were suspended at OD$_{600}$ = 0.2 in 10 mL Graver Wade media (Wade and Graver, 2007) in 125 mL
sterile flasks and grown for 4.5h at 37°C and 5% CO₂ in a shaker incubator. Bacteria were removed by collection of culture supernatant after centrifugation followed by filtration through a sterile 0.2μm syringe filter. The viability (CFU/mL) was monitored by plating three dilutions of a sample from each strain on GCB agar plates at the beginning and end of each incubation and counting colonies after 48h. Culture supernatants were generated on multiple days to account for day-to-day variability.

2.3.3 Culture supernatants

*N. gonorrhoeae* strains were grown overnight on GCB agar plates, suspended at OD<sub>600</sub> = 0.2 in 10 mL Graver Wade media, and grown for 4.5h at 37°C and 5% CO₂ in a shaker incubator. The bacterial density (CFU/mL) was monitored by counting colonies on plated dilutions of the bacterial suspensions made before and after the growth period. Culture supernatants were generated by centrifugation followed by filtration through a sterile 0.2μm filter on at least three separate days to account for day-to-day variability.

2.3.4 Reporter cell lines

Commercially available HEK 293 cells stably transfected with the NOD1, NOD2, or TLR2 receptor and an alkaline phosphatase reporter (Invivogen) were stimulated with FA1090 wildtype, Δ*lgtD* or Δ*ltgA*/Δ*lgtD* conditioned media or isolated PGN. Cells were plated in 96-well plates according to manufacture specification and incubated with samples at 37°C, 5% CO₂ overnight. Receptor response was quantified using a colorimetric alkaline phosphatase assay.

2.3.5 Cell Culture and Cytokine Analysis

THP1 cells were grown in suspension in RPMI 1640 containing 10% fetal bovine serum, 50 U/mL penicillin, and 50 μg/mL streptomycin. Human dendritic cells were generated by
culture of CD34+ cells from peripheral blood in AIM V medium with 10% human AB serum and Stem Cell Factor (SCF 50 ng/mL), Flt3L (100 ng/mL), GM-CSF(800 U/mL) and IL-4(500 U/mL) for 14 days. Blood was obtained from subjects enrolled in a UNC IRB approved study (Study #05-2860) after obtaining informed consent. The cells were cultured and de-identified prior to transfer to our laboratory for exposure to N. gonorrhoeae culture supernatant (Zhu et al., 2012). The use of these de-identified cells was reviewed by the UNC Office of Human Research Ethics (Study #12-0024) and was determined not to require further IRB approval because the study did not constitute human subjects research as defined under federal regulations [45 CFR 46.102 (d or f) and 21 CFR 56.102(c)(e)(l)]. THP1 cells were plated at 1 × 10^6 cells/mL and exposed to either N. gonorrhoeae culture supernatants or live N. gonorrhoeae for 4h. In the cases in which cells were exposed to live bacteria, antibiotic-free RPMI 1640 containing 10% fetal bovine serum only was used. TNF-α and IL-1β cytokine analyses on cell supernatants were done using ELISA (BD Biosciences).

### 2.3.6 Purification of N. gonorrhoeae peptidoglycan fragments

Peptidoglycan fragments from N. gonorrhoeae were isolated by the Dillard Lab as described previously(Cloud-Hansen et al., 2008). Briefly, fragments were obtained from PGN sacculi isolated from ΔpacA ΔmsbB N. gonorrhoeae. PGN fragments containing the 1,6-anhydro bond were obtained by digesting whole N. gonorrhoeae PGN with soluble LtgA for 15min or 4h to obtain, multimers and dimers, over overnight at 37°C to obtain monomers. Monomeric fragments containing the reducing bond were obtained by digesting whole PGN with human neutrophil lysozyme overnight at 37°C. Purification of soluble multimer, dimer, and monomer fragments was performed on tandem 350-ml Bio-Gel P6 and Bio-Gel P30 size exclusion columns and tripeptide and tetrapeptide monomers were further purified by HPLC.
Peptidoglycan structures were confirmed by mass spectroscopy. Peptidoglycan fragments were quantified using a Fluoraldehyde OPA (o-phthalaldehyde) Reagent solution (Thermo Scientific Pierce). Fluorescent units correlate with the number of primary amines, a single primary amine which is present on the diaminopimelic acid of a monomeric PGN unit. Peptidoglycan solutions were standardized for experimental use using isoleucine and phenylalanine.

2.4 Results

2.4.1 The *N. gonorrhoeae* LtgA and LtgD proteins play a role in suppressing the inflammatory cytokine production.

In order to assess the effect of *N. gonorrhoeae* LtgA and LtgD on host cell cytokine production, PMA-stimulated THP1 cells were exposed to wild type *N. gonorrhoeae* strain FA1090 or an isogenic mutant with deletions of the *ltgA* and *ltgD* genes (FA1090 Δ*ltgA/ΔltgD*) and the accumulation of IL-1β and TNF-α in the cell culture supernatants was measured. Cells exposed to FA1090 Δ*ltgA/ΔltgD* secreted significantly greater quantities of both cytokines than those exposed to the isogenic parent (Figure 2.1A). Culture supernatants from FA1090 Δ*ltgA/ΔltgD* also elicited greater inflammatory cytokine production from THP1 cells and primary human dendritic cells than culture supernatant from the parental strain (Figure 2.1B and 2.1C). To ensure the enhanced induction of host cell cytokine production by bacteria or bacterial culture supernatants was not due to differences in the growth of wild type and Δ*ltgA/ΔltgD* FA1090 strains, the optical density of the cultures was monitored over a four-hour growth period and no significant difference in the optical density was observed between strains (Figure 2.1D). Profiling of PGN fragment release from wild type and Δ*ltgA/ΔltgD* FA1090 by size exclusion chromatography of culture supernatant after metabolic labeling of the bacteria with [6-\(^3\)H]glucosamine revealed that Δ*ltgA/ΔltgD* FA1090 released dimeric and multimeric PGN while
wild type FA1090 released primarily monomeric PGN with a small peak of dimeric PGN (Figure 2.1E), as previously described for *N. gonorrhoeae* strain MS11 (Cloud-Hansen et al., 2008). Overall, these data demonstrate that culture supernatants containing multimeric PGN fragments released from ΔltgA/ΔltgD *N. gonorrhoeae* have enhanced capacity to elicit host inflammatory cytokine production.

### 2.4.2 The *N. gonorrhoeae* LtgA and LtgD proteins modulate NOD2 and TLR2 receptor signaling.

To identify immune receptors involved in increased host cytokine response to FA1090 ΔltgA/ΔltgD, commercially available HEK293 reporter cells stably transfected with NOD1, NOD2, TLR2, TLR4, or TLR9 were used to assess innate immune receptor activation by culture supernatants from wild type or ΔltgA/ΔltgD *N. gonorrhoeae*. FA1090 ΔltgA/ΔltgD culture supernatants consistently resulted in greater NOD2 activation (Figure 2.2A), with a 13-fold increase in activation over basal levels, compared to a 5-fold increase for the wild type. Similarly, culture supernatants from FA1090 ΔltgA/ΔltgD induced a 7.5-fold activation of reporter activity in TLR2-expressing reporter cell lines, compared to the 4.5-fold increase caused by wild type culture supernatants (Figure 2.2B). In contrast, NOD1-, TLR4-, and TLR9-expressing reporter cells demonstrated equivalent reporter activation after exposure to either FA1090 or FA1090 ΔltgA/ΔltgD culture supernatants (Figure 2.2C, 2.2D, and 2.2E). HEK293 cells carrying only the reporter demonstrated there was no reporter activation difference between FA1090 and FA1090 ΔltgA/ΔltgD culture supernatants due to stimulation of endogenous innate immune receptors (Figure 2.2F).
2.4.3 Monomeric and dimeric peptidoglycan fragments from *N. gonorrhoeae* differentially stimulate innate immune receptors.

Because culture supernatants from ΔltgA/ΔltgD *N. gonorrhoeae* contain decreased levels of anhydro PGN monomers and increased levels of multimeric PGN (Figure 2.1G), we sought to test whether polymeric *N. gonorrhoeae* PGN exhibited a differential capacity to stimulate NOD2 and TLR2 when compared to monomeric PGN released through the activity of LtgA. Soluble monomeric and multimeric PGN was generated by complete or partial digestion of *N. gonorrhoeae* sacculi with recombinant LtgA. The quantity of PGN in the preparations was assessed by quantitating the free amine group of the meso-DAP and equivalent quantities of DAP mass were used to test the ability of monomeric and multimeric PGN to stimulate NOD1, NOD2, and TLR2 using HEK293 reporter cell lines. Both monomeric PGN and multimeric PGN induced significant NOD1 activation; monomeric PGN induced an 8-fold activation of alkaline phosphatase activity above basal levels and equivalent quantities of multimeric PGN induced 2.7-fold levels of reporter activation (Figure 2.3A). In contrast, incubation of the cells with multimeric PGN induced significant activation of NOD2 (3.3-fold induction) while incubation with monomeric PGN did not (1.3-fold induction, p>0.05) (Figure 2.3B).

The purified PGN fragments showed modest activation of TLR2 above baseline with no significant difference between monomeric and multimeric PGN (Figure 2.3C). The stimulation observed in NOD1-, NOD2-, and TLR2-expressing cells was not observed in cells expressing only the reporter construct (Figure 2.3D) Because we did not observe differences between the different PGN species’ capacity to stimulate TLR2, we sought to determine if other known activators of TLR2 were differentially present in the culture supernatants of FA1090 and FA1090 ΔltgA/ΔltgD. Immunoblot analysis with antibodies directed against the *N. gonorrhoeae*
PorB and the lipoprotein Lip, both known to activate TLR2, showed that both proteins were in higher abundance in culture supernatants from FA1090 ΔltgA/ΔltgD than those from the parental FA1090 strain (Figure 2.3E) (Fisette et al., 2003; Massari et al., 2009). Overall, these data indicate that isolated gonococcal PGN, in either multimeric or 1,6-anhydro-MurNAc-containing monomeric form, is a weak stimulator of TLR2 and that LtgA and LtgD may modulate host TLR2 signaling by reducing release of TLR2 activating outer-membrane proteins.

2.4.4 N. gonorrhoeae LtgA-generated PGN monomers have diminished capacity to activate NOD2 compared to host lysozyme-generated PGN monomers.

Because NOD2 can be stimulated by monomeric MDP, the increased NOD2 signaling induced by multimeric PGN was unlikely to be attributable simply to the multimeric structure of the PGN. Instead, we hypothesized that reducing MurNAc-containing PGN monomers produced by the action of host lysozyme on multimeric PGN could stimulate NOD2 while 1,6-anhydro-MurNAc-containing monomeric PGN generated by LtgA could not (Figure 2.4A). To test this, we generated PGN monomers from N. gonorrhoeae PGN using either recombinant LtgA or human neutrophil lysozyme. Structures of the resulting monomers were confirmed by mass spectrometry and show PGN monomers with peptide chain lengths of two to five amino acids (data not shown). Isolated monomeric fragments were tested for activation of NOD1 and NOD2 (Figure 2.4B and 2.4C). Because the PGN monomers should contain equivalent quantities of the NOD1-stimulating iE-DAP ligand, irrespective of the enzyme used to cleave the GlcNAc and MurNAc bond, we did not expect to observe a difference in NOD1 activation between monomer preparations. As expected, these PGN monomers stimulated NOD1 receptor activation with equal potency (Figure 2.4B). However, in NOD2-expressing reporter cells, lysozyme-digested PGN monomers were more potent activators of NOD2 signaling when compared to LtgA-
digested PGN monomers. To ensure that the reduced NOD2 signaling was the result of the presence of the 1,6-anhydro-MurNAc moiety of the monomer and not variability in amino acid chain lengths within the mixture, tripeptide- and tetrapeptide-containing, 1,6-anhydro-muramyl or reducing-muramyl PGN monomers were isolated by HPLC and tested using NOD1- or NOD2- expressing reporter cell lines. As expected, there was no difference in NOD1 activation between the 1,6-anhydro- or reducing-MurNAc PGN tripeptide at either concentration tested. The 1,6-anhydro- and reducing-MurNAc monomers containing tetrapeptide induced little to no activation of NOD1 (Figure 2.4D). 1,6-anhydro-MurNAc-containing PGN tripeptide monomer, the predominant PGN monomer released by N. gonorrhoeae, did not activate NOD2 above basal levels. In contrast, the reducing-MurNAc tripeptide monomer produced by human lysozyme induced a 10.8-fold (8 uM) and 3.9-fold (800 nM) increase in NOD2 activation (Figure 2.4E). Additionally, 1,6-anhydro-MurNAc-containing PGN tetrapeptide (TCT) failed to activate NOD2, while the reducing-MurNAc-containing PGN tetrapeptide (8 uM) also activated NOD2, a 8.2-fold increase over basal levels. These data demonstrate that PGN monomers released by N. gonorrhoeae lytic transglycosylases LtgA and LtgD fail to activate host cell NOD2 because the 1,6-anhydro-MurNAc structure in the monomer is not recognized by NOD2.

2.5 Discussion

The role of PGN monomer production and release by N. gonorrhoeae in host immune recognition of the bacteria is not well studied. Previous studies have demonstrated that N. gonorrhoeae releases PAMPs that preferentially induce NOD1 activation over NOD2 activation (Mavrogiorgos et al., 2013). Our data show that an important factor in directing the host innate immune response to N. gonorrhoeae are the LtgA and LtgD lytic transglycosylases, which act in the production of 1,6-anhydro monomeric PGN fragments (Summarized in Figure 2.5).
In agreement with previously published reports, our studies demonstrate that endogenous PGN monomers produced by *N. gonorrhoeae* LtgA and LtgD are potent activators of NOD1 but not NOD2. This activation of NOD1, but not NOD2, by monomeric anhydro PGN agree with previous reports that TCT does not activate human NOD2 (Magalhaes et al., 2005) but that PGN structures terminating with iE-DAP in the peptide chain can activate NOD1 (Girardin et al., 2003c). Our data in NOD2-expressing reporter cells show that the multimeric forms of PGN are more potent than the monomeric form. The capacity of multimeric forms of PGN to stimulate NOD2 may in part be due to digestion of these PGN fragments by host cellular lysozyme, which has previously been shown to facilitate NOD2 recognition of *Streptococcus pneumonia* (Davis et al., 2011). Lysozyme generated PGN monomers are more potent activators of NOD2 than the LtgA 1,6-anhydro product regardless of the amino acid chain length. A synthetic addition of UDP to the hydroxyl group on the muramic acid has been previously shown to enhance NOD2 activation to a synthetic *meso*-DAP containing muramyl tripeptide, demonstrating the importance of the muramic acid moiety of PGN in NOD2 mediated recognition of these PAMPs (Girardin et al., 2003c). We now demonstrate that modification to the muramic acid sugar by a native bacterial enzyme can alter NOD2 signaling in response to the bacteria. In this case, the formation of PGN monomers with a 1,6-anhydro bond by LtgA reduces stimulation of NOD2, which is otherwise triggered by secreted multimeric PGN fragments generated by bacterial growth. Together, these data help explain why an increase in NOD2, but NOD1, signaling is seen in response to culture supernatants from FA1090 ΔltgA/ΔltgD, which produce large PGN fragments that can be processed by native lysozyme to produce the NOD2-stimulating, reducing PGN monomer. These data present an explanation for a recently reported observation that *N.*
*N. gonorrhoeae* bacterial culture supernatants induced weak NOD2 activation when compared to whole bacteria lysates (Mavrogiorgos et al., 2013).

In addition to inducing more robust host NOD2 signaling, the deletion of *ltgA* and *ltgD* in *N. gonorrhoeae* leads to increased TLR2 signaling in response to the bacteria. Previous studies have implicated TLR2 in host response to PGN while some studies suggest that TLR2 has no direct ability to recognize PGN (Li et al., 2010; Travassos et al., 2004). It has been proposed that TLR2 responds to multimeric PGN but not monomeric PGN (Müller-Anstett et al., 2010). Others have proposed that PGN signaling via TLR2 is the result of contaminants, such as lipoproteins or teichoic acids (Volz et al., 2010). Our results do not show a significant difference in the activation of TLR2 between multimeric PGN, dimeric PGN, or monomeric PGN, though all three PGN species only weakly activate TLR2. The increased capacity to induce TLR2 activation by culture supernatants from the Δ*ltgA/ΔltgD* mutant is accompanied by increased quantities of known TLR2 stimulating proteins from *N. gonorrhoeae*, PorB and Lip (Massari et al., 2002). We hypothesize that release of these TLR2-activating polypeptides accompanies release of larger PGN multimers that are not processed into 1,6-anhydro-MurNAc-containing PGN monomers in this strain.

Thus we present here a specific modification of PGN by *N. gonorrhoeae* lytic transglycosylases LtgA and LtgD suppresses the response of the innate immune receptor NOD2, in contrast to the modification of PGN generated during break-down by host lysozyme. Interestingly, peptidoglycan modification via O-acetylation has previously been shown to increase PGN resistance to lysozyme digestion in many pathogenic bacteria, including *N. gonorrhoeae* (Bera et al., 2006; Dillard and Hackett, 2005; Moynihan and Clarke, 2011; Rosenthal et al., 1982, 1983). The PGN O-acetyltransferase in *Staphylococcus aureus* has been
shown to modulate host cytokine responses to the bacteria by modulation of signaling by the innate immune receptors NOD2 and NLRP3 (Shimada et al., 2010). Together, these data combined with our reported findings suggest that the *N. gonorrhoeae* maintains multiple mechanisms to suppress host NOD2 signaling. Interestingly, while suppressing host NOD2 signaling, *N. gonorrhoeae* secretes large quantities of NOD1 activating ligand, suggesting the two receptors play non-redundant roles in gonococcal pathogenesis. This presents a challenge to study *N. gonorrhoeae* pathogenesis using the murine model of *N. gonorrhoeae* due to the differences between mouse and human NOD1 ligand specificity (Magalhaes et al., 2005). It is yet to be determined if the ability of these PGN fragments to avoid NOD2 signaling while simultaneously stimulating NOD1 is beneficial to the establishment, the persistence and/or transmission of infection. Activation of NOD2 by MDP has been shown to stimulate production of antimicrobial peptides like HNP1 and β-defensin-2 (Voss et al., 2006; Yamamoto-Furusho et al., 2010). NOD2 has been shown to play a role in adaptive immune responses that are known to be weak or absent in humans infected with *N. gonorrhoeae* (Fox et al., 1999). For example, NOD2 is known to mediate MHC class II antigen-specific CD4+ responses and facilitate polarization of Th2 cells in response to those antigens (Cooney et al., 2009; Magalhaes et al., 2008). In the mouse model of *N. gonorrhoeae*, induction of Th2 responses have been shown to be critical in the clearance of *N. gonorrhoeae* (Liu and Russell, 2011; Liu et al., 2012). Suppression of NOD2 activation by *N. gonorrhoeae* LtgA and LtgD modified PGN may therefore may be mechanisms to suppress both innate and adaptive immune responses to this pathogen, allowing for the persistence of infection and transmission of the disease. Given the
role of NOD2 in host defense and immune response, the modification of released PGN fragments should be further investigated for their potential role in the host immune response to *N. gonorrhoeae*. 
IL-1β (top) and TNF-α (bottom) production was measured in (A) PMA-stimulated THP1 cells challenged with live *N. gonorrhoeae* (Multiplicity of Infection = 0.1), THP1 cells (B) or human dendritic cells (C) exposed to *N. gonorrhoeae* culture supernatants from *N. gonorrhoeae* grown in Graver-Wade medium as described in the Experimental Procedures. Secreted cytokines were below the level of detection (15.6 pg/ml for IL-1β and 78 pg/ml for TNF-α) for untreated cells or cells treated with Graver-Wade medium. (D) Optical density of indicated *N. gonorrhoeae* strain cultures at the indicated time points (E) Size exclusion chromatography profiles of PGN fragments released from FA1090 or FA1090 ΔltgA/ΔltgD after labeling of PGN pool with [6-3H]glucosamine. Data points are plotted as mean +/- S.E.M. from triplicate samples. Plots are representative of repeated experiments (for A-B, n>3 for C-E, n=2) Significance was determined using Student’s T-test with the determined p-value indicated.

Figure 2.1. Deletion of the *ltgA* and *ltgD* genes in *N. gonorrhoeae* results in increased inflammatory signaling in human monocytes and dendritic cells.
Figure 2.2. Culture supernatants from *N. gonorrhoeae* lacking *ltgA* and *ltgD* genes exhibit enhanced activation of human NOD2 and TLR2, but not NOD1.

(A) NOD2, (B) TLR2, (C) NOD1, (D) TLR4, (E) TLR9, and (F) Null HEK293-Blue™ cells were treated with culture supernatants from the indicated *N. gonorrhoeae* strains and reporter SEAP activity was measured as described in the Experimental Procedures. Data is expressed as fold activation over the basal levels of alkaline phosphatase production from cells treated with Graver-Wade Medium for each independent experiment. Data shown represent mean values +/- S.E.M. from cells treated with two preparations of culture supernatants and experiments were repeated at least twice. Significance was determined using ANOVA with Bonferroni posttest for multiple comparisons, a corrected p-value < 0.05 was considered significant.
Monomeric PGN was prepared using recombinant LtgA (as described in Experimental Procedures) and receptor activation was compared to multimeric PGN using reporter cell lines described in Figure 2.2: (A) NOD1, (B) NOD2, (C) TLR2, and (D) Null1 HEK293-Blue™ cells. (E) Culture supernatants from the indicated strains of *N. gonorrhoeae* were analyzed by immunoblot using antibodies directed against PorB (top) and lipoprotein Lip (bottom). (A-D) Data shown represent mean values +/- S.E.M. from cells treated with two preparations of culture supernatants and experiments were repeated at least twice. Statistical analysis was done using one-way ANOVA with Bonferroni posttest for multiple comparisons and indicates comparison to basal activation (* p< 0.05; *** p<0.001). The dashed line demonstrates the normalized basal SEAP activity.

Figure 2.3. Multimeric PGN and Monomeric PGN released by *N. gonorrhoeae* LtgA differentially activate NOD1 and NOD2.
Figure 2.4 PGN digested by LtgA but not human neutrophil lysozyme escapes detection by NOD2.

(A) The structure of 1,6-anhydro-MurNAc-containing PGN monomers predominantly released by LtgA (left) and reducing-MurNAc-containing PGN monomers predominantly released by lysozyme (right). (B) NOD1- and (C) NOD2-expressing reporter cells were treated with the indicated LtgA- or lysozyme-liberated PGN monomers at the indicated concentrations. Monomers contained a mixture of PGN monomer species as described. The indicated PGN monomer species were further isolated using HPLC and the capacity of each to stimulate NOD1 (D) and NOD2 (E) reporter cells was assessed. Data shown are representative of at least two experiments from at least two independent preparations of PGN monomers. (B-E) Data shown represent mean values +/- S.E.M. from cells treated with two preparations of culture supernatants and experiments were repeated at least twice. Statistical analysis was done using one-way ANOVA with Bonferroni posttest for multiple comparisons (**** P < 0.0001). The dashed line demonstrates the normalized basal SEAP activity.
Figure 2.5. Summary figure showing *N. gonorrhoeae* evasion of NOD2 signaling through the production of 1,6-anhydro PGN monomers by LtgA and LtgD.

Wild type *N. gonorrhoeae* release large quantities of cytotoxic 1,6-anhydro PGN monomers during growth. These monomers are produced through the action of lytic transglycosylases LtgA and LtgD. The addition of the 1,6-anhydro bond on the muramic acid sugar by LtgA and LtgD results in PGN fragments that cannot be recognized by NOD2. When the *ltgA* and *ltgD* genes are deleted, *N. gonorrhoeae* release PGN multimers. These multimers are then subject to digestion by host lysozyme and detection by NOD2.
Chapter 3

The role of \textit{N. gonorrhoeae} peptidoglycan in pathogenesis using a murine model of infection

3.1 Overview

\textit{Neisseria gonorrhoeae} releases 1,6-anhydro peptidoglycan monomers during growth that are the product of two lytic transglycosylases, LtgA and LtgD. \textit{N. gonorrhoeae} lacking LtgA and LtgD do not release peptidoglycan monomers, but rather large, multimeric peptidoglycan fragments. We sought to determine the role of released peptidoglycan monomers in the pathogenesis of gonorrhea infection \textit{in vivo} in a murine model of infection using a \textit{N. gonorrhoeae} mutant with the \textit{ltgA} and \textit{ltgD} genes deleted. Secondly, as we have previously shown that LtgA and LtgD reduce NOD2 activation \textit{in vitro}, we also wanted to assess the role of NOD2 in pathogenesis. In this study, we show that wild type mice that are infected with FA1090 Δ\textit{ltgA}/Δ\textit{ltgD} have a lower bacterial burden compared to mice infected with wild type FA1090 and that FA1090 Δ\textit{ltgA}/Δ\textit{ltgD} has a fitness defect in a competitive infection model. Deletion of \textit{ltgA} and \textit{ltgD} did not affect the persistence of infection in mice. Finally, NOD2 does not appear to play a role in pathogenesis in the mouse model of infection, as no difference between bacterial burden or infection persistence was seen in \textit{Nod2}⁻⁻ mice infected with FA1090 compared to wild type mice.

3.2 Introduction

\textit{Neisseria gonorrhoeae} is a gram-negative pathogen that is the causative agent for the sexually transmitted infection gonorrhea. During symptomatic infection in humans, \textit{N.}
*N. gonorrhoeae* stimulates the production of inflammatory cytokines IL-17A and IL-23 that leads to the influx of polymorphonuclear leukocytes (PMNs) (Gagliardi et al., 2011). This response is thought to be directed by IL-17 producing Th17 cells, which is observed in the murine model of infection (Feinen et al., 2010). *N. gonorrhoeae* does not induce a protective adaptive immune response in the host. Antibody titers of anti-gonococcal antibodies in patients with re-infection are no higher than in patients with first time infection, and re-infection can occur with the same *N. gonorrhoeae* serovar (Fox et al., 1999; Hedges et al., 1999; Hobbs et al., 1999). In the mouse model of infection, *N. gonorrhoeae* has been shown to suppress a protective Th1/Th2 response through the induction of TGF-β (Liu et al., 2012). The mechanism by which TGF-β induction occurs is unknown.

During growth, *N. gonorrhoeae* release large quantities of cytotoxic anhydro peptidoglycan monomers. PGN monomers released by *N. gonorrhoeae* contain one *N*-acetylglucosamine-1,6-anhydro-*N*-acyetylmuramic acid disaccharide unit linked to a peptide, 80% of which are the L-alanine-D-glutamic acid-*meso*-diaminopimelic acid tripeptide, with the remaining 20% composed mostly of terminal D-alanine containing tetrapeptide (Sinha and Rosenthal, 1980). The tetrapeptide PGN monomer is structurally identical to the PGN monomers released by *Bordetella pertussis*, known as tracheal cytotoxin due to their ability to kill ciliated epithelial cells (Cookson et al., 1989; Goldman et al., 1982). PGN monomers from *N. gonorrhoeae* have been shown to damage fallopian tube explants and induce inflammatory cytokine production (Chan et al., 2012; Melly et al., 1984). It is hypothesized that this tissue damage may be important in the establishment of infection and transmission, though the effects of cytotoxic peptidoglycan monomers on cervical and urethral epithelium have not been studied.
The full extent to which *N. gonorrhoeae* PGN monomer release plays a role in pathogenesis and immune recognition of the bacteria is unknown.

Inflammatory signaling in response to bacterial infections is mediated by the detection of pathogen-associated molecular patterns (PAMPs) through numerous extracellular and intracellular receptors. The innate immune nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and 2 (NOD2) proteins are cytosolic sensors of bacterial peptidoglycan (PGN). Activation of NOD1 by PGN peptide chain component D-glutamyl-meso-diaminopimelic acid (iE-DAP) in gram-negative bacteria and NOD2 by the muramyl dipeptide (MDP) moiety found in the PGN of most bacteria leads to the induction of NF-κB and inflammatory cytokine production (Chamaillard et al., 2003; Girardin et al., 2003a, 2003b; Inohara, 2003). Both NOD1 and NOD2 have shown to play a role in directing the immune response to numerous pathogenic bacteria. NOD1 has been shown to play a critical role in controlling the infection to *Helicobacter pylori*, as *Nod1−/−* mice were more susceptible to gastric infection. This effect was dependent on PGN delivery to host cells through the action of the *H. pylori* type IV secretion system (Viala et al., 2004). *In vitro*, NOD2 has been shown to play a role in controlling the growth of *Mycobacterium tuberculosis* in human monocyte-derived macrophages and primary mononuclear cells (Brooks et al., 2011; Ferwerda et al., 2005). Survival of *Nod2−/−* mice challenged with intraperitoneal *Staphylococcus aureus* was significantly reduced compared to wild type mice (Deshmukh et al., 2009). In mouse neutrophils, NOD2, but not NOD1, directed the production of innate immune cytokines in response to several gram negative bacteria (Jeong et al., 2014). NOD1 and NOD2 signaling in response to *N. gonorrhoeae* has recently been a topic of investigation. Culture supernatants from *N. gonorrhoeae* robustly activate NOD1 and whereas activation of NOD2 is significantly less
(Mavrogiorgos et al., 2013). This is likely due to the presence of 1,6-anhydro PGN monomers that are released into the supernatant of growing *N. gonorrhoeae*, which contain the iE-DAP moiety capable of activating NOD1 but are poorly activating of NOD2 due to the presence of the 1,6-anhydro bond on the muramic acid sugar (Chapter 2, Section 2.4). These PGN monomers are produced through the action of two lytic transglycosylases, LtgA and LtgD. *N. gonorrhoeae* with the *ltgA* and *ltgD* genes deleted release multimeric PGN fragments rather than monomers (Cloud-Hansen et al., 2008).

In this study we sought to determine the role of both NOD2 and the *ltgA* and *ltgD* genes in *N. gonorrhoeae* pathogenesis using the murine model of infection. We found that *N. gonorrhoeae* lacking PGN processing by LtgA and LtgD had diminished bacterial burdens over the course of infection. A *N. gonorrhoeae* mutant with the *ltgA* and *ltgD* genes deleted, FA1090 ∆*ltgA*/∆*ltgD*, was impaired in competitive infections. This indicates that the multimeric PGN released by these bacteria, which is capable of activating NOD2 more efficiently than PGN released from wild type *N. gonorrhoeae*, did not impact survival of the isogenic parent FA1090. In concordance with this, mice lacking NOD2 were not impaired in their ability to clear *N. gonorrhoeae*, suggesting that NOD2 signaling is not important in acute *N. gonorrhoeae* infection, at least in the mouse vaginal infection model.

### 3.3 Materials and Methods

#### 3.3.1 Bacterial strains

For the competitive co-infections, FA1090 and FA1090 ∆*ltgA*/∆*ltgD* were transformed with pCTS32 (Steichen et al., 2008) by spot transformation on GC agar as described previously to generate spectinomycin-resistant variants of each strain (Johnston and Cannon, 1999). These strains were designated FA1090-spectinomycin and FA1090 ∆*ltgA*/∆*ltgD*-spectinomycin.
N. gonorrhoeae strain FA1090 ΔltgA/ΔltgD (JD) was generated as described previously (Cloud and Dillard, 2002; Cloud-Hansen et al., 2008). Whole genome sequencing of FA1090 ΔltgA/ΔltgD (JD) confirmed that the entire coding region of ltgD was deleted while the ltgA gene was disrupted by deletion of the last 1420 bp of the 1850 bp ltgA coding region and insertion of the ermC gene conferring erythromycin resistance. A second construction of the ΔltgA/ΔltgD mutant, FA1090 ΔltgA/ΔltgD (KK) is described in Appendix 1.

3.3.2 Experimental murine infection with N. gonorrhoeae

Female BALB/c (4 to 6 weeks old) were purchased from The Jackson Laboratories, ME. BALB/c Nod2−/− mice were a gift from Holly Rosenzweig (Rosenzweig et al., 2011) and age-matched female BALB/c Nod2−/− mice were obtained through in-house breeding. Mice in the diestrus stage of the estrus cycle were started on a regimen of subcutaneous water-soluble 17β-estradiol (0.5mg) two days prior to infection, the day of infection, and two days following infection to promote susceptibility to N. gonorrhoeae (Song et al., 2008). Mice additionally received oral trimethoprim sulfate (0.04mg/mL), streptomycin sulfate (5mg/mL), and vancomycin (250μg/mL) in addition to intraperitoneal streptomycin sulfate and vancomycin twice daily (3.6mg and 0.6mg, respectively). On the day of infection, mice were infected with Opa-matched 1x10^6 CFU FA1090 or FA1090 ΔltgA/ΔltgD. Vaginal mucus was collected daily with sterile swabs to assess commensal colonization and colonization with N. gonorrhoeae. The bacterial load for each mouse was calculated by performing serial 10-fold dilutions of the vaginal samples in a 96-well plate and replica plating in at least triplicate onto GC-VCNTS agar.

3.3.4 Competitive co-infection in mice

Mice in the diestrus stage of the estrus cycle were selected for the study and made susceptible to N. gonorrhoeae with the administration of water-soluble 17β-estradiol and an oral
and intraperitoneal regimen of antibiotics as described above. BALB/c mice were inoculated vaginally with equal CFU/mL FA1090-spectinomycin and FA1090 ΔltgA/ΔltgD or FA1090 and FA1090 ΔltgA/ΔltgD-spectinomycin for a total of 2x10^6 CFU per mouse. Bacterial burden in each mouse was assessed using vaginal swabs as described above, and *N. gonorrhoeae* were replica plated on GC-VNTCS-spectinomycin and GC-VNTS agar. Total burden was calculated from colonies counted on GC-VNTS agar. The amount of bacteria without spectinomycin resistance was determined by subtracting the amount of *N. gonorrhoeae* recovered from the GC-VNTCS-spectinomycin agar from total bacteria burden as calculated from GC-VNTS agar. The presence of a spectinomycin resistance marker did not alter the ability of either FA1090 or FA1090 ΔltgA/ΔltgD to colonize mice relative to the isogenic *N. gonorrhoeae* strains without spectinomycin resistance.

3.3.3 Characterization of inflammation

The percentage of neutrophil influx was assessed by microscopic examination of stained vaginal smears. Images were taken of at least three fields and the number of cornified epithelial cells, nucleated epithelial cells, and neutrophils were counted on each. Data are expressed as a percentage of neutrophils relative to total cells in the field.

3.3.4 Cytokine analysis of mouse derived dendritic cells

Bone marrow was harvested from the femurs of euthanized mice and the cells were grown for 7 days in RPMI 1640 containing 10% inactivated FBS, penicillin/streptomycin, GM-CSF, and IL-4. The phenotype of BMDCs grown under these conditions has been verified via flow cytometry using cell markers CD11c and MHC II, and costimulation markers CD80, CD86, and CD40. Cells were exposed to either live *N. gonorrhoeae* or *N. gonorrhoeae* culture supernatants
generated as described previously (Chapter 2, Section 2.3.3.) for 16 hours. Supernatants were collected and cytokines were measured by ELISA (BD Scientific).

3.4 Results

3.4.1 Mice infected with ΔltgA/ΔltgD *N. gonorrhoeae* have a lower bacterial burden compared to infection with wild type

To assess the role of *ltgA* and *ltgD* in infection pathogenesis, female BALB/c mice were infected with either FA1090 ΔltgA/ΔltgD or wild type FA1090. Vaginal samples were collected in PBS daily for ten days to monitor bacterial burden. Samples were diluted serially 10-fold and replica plated onto GC-VNTCS agar. Colonies at each dilution were counted to establish CFU/mL for each mouse on each day. Bacteria recovered (CFU/mL) from infected mice on each day for mice infected with FA1090 or FA1090 ΔltgA/ΔltgD is shown in Figure 3.1A. Area under the curve (AUC) for each infected mouse was calculated, and showed that the overall bacterial burden in mice infected with FA1090 ΔltgA/ΔltgD was significantly lower than mice infected with FA1090 *N. gonorrhoeae* (Figure 3.1B).

3.4.2 Deletion of *ltgA* and *ltgD* does not impact infection persistence *in vivo*

The persistence of *N. gonorrhoeae* FA1090 or FA1090 ΔltgA/ΔltgD in BALB/c mice was monitored over the course of ten days. Mice that were not positive for *N. gonorrhoeae* culture on day 10 were considered to have cleared the infection on the day after the last day detectable levels of *N. gonorrhoeae* were recovered from vaginal swabs. Mice in which *N. gonorrhoeae* was never recovered during the 10 days of monitoring were considered to not have been infected. Deletion of *ltgA* and *ltgD* may play a role in early infection, as there was a statistically significant difference in persistence of infection up to day 4 (Figure 3.2). However, this may simply reflect the low bacterial burden characteristic of FA1090 ΔltgA/ΔltgD that was frequently
below the lower limit of detection of the assay, as demonstrated in some mice by several days of no *N. gonorrhoeae* recovery followed by at least one day of positive culture. Over the total course of 10 days there was not a statistically significant difference in the persistence of infection between FA1090 and FA1090 ΔltgA/ΔltgD.

### 3.4.3 Inflammatory responses to FA1090 ΔltgA/ΔltgD in mice

We measured inflammatory cytokine response in mouse bone marrow derived dendritic cells (BMDCs) to determine if mouse cells would respond similarly to *N. gonorrhoeae* FA1090 and FA1090 ΔltgA/ΔltgD at human THP1 and primary blood dendritic cells discussed in Chapter 2 (Figure 2.1A-C). Mouse BMDCs were exposed to *N. gonorrhoeae* culture supernatants and IL-1β and TNF-α secretion was measured in the supernatants. As with the human cells lines tested previously, mouse BMDCs produced more inflammatory cytokines IL-1β and TNF-α in response to culture supernatants from FA1090 ΔltgA/ΔltgD compared to wild type FA190 (Figure 3.3A).

We assayed mice infected with *N. gonorrhoeae* for inflammation by counting the number of neutrophils relative to other cells present on slides of vaginal swabs. In experimental mice that *N. gonorrhoeae* was never recovered from, no neutrophils were observed. In mice that were positive for *N. gonorrhoeae* infection, 3/7 mice infected with FA1090 were positive for neutrophil influx on at least one day and in mice infected with FA1090 ΔltgA/ΔltgD (KK) (construction described in Appendix 1) 1/6 mice were positive for neutrophil influx. Neutrophil influx on average was higher in FA1090-positive mice, with a mean of 3%, 5%, and 2% on days 5, 6, and 7 respectively, though there was considerable variation between mice. In FA1090 ΔltgA/ΔltgD-positive mice, the mean neutrophil influx was 0%, 0%, and 0.2% for days 5, 6, 7 respectively (Figure 3.3B). Thus while FA1090 ΔltgA/ΔltgD does induce influx of PMNs,
inflammation is less than FA1090, which does not recapitulate the increased inflammatory cytokine release by isolated dendritic cells or monocyte cell lines that we have observed in cell culture-based experiments. However, bacterial loads in mice infected with FA1090 ΔltgA/ΔltgD were lower than mice infected with FA1090, and sample numbers were low for each group.

3.4.4 The ΔltgA/ΔltgD N. gonorrhoeae mutant has a fitness defect in competitive infection in vivo

To evaluate whether or not the release of PGN multimers, rather than monomers, could act on the host in trans to reduce gonococcal colonization by both wild type and mutant FA1090, we performed a competitive co-infection with FA1090 in wild type BALB/c mice. If FA1090 ΔltgA/ΔltgD is defective in single infection when compared to wild type FA1090, but not defective when compared to wild type in competitive infection, it could suggest that either the multimeric PGN acts in trans to reduce survival of FA1090 or that secretion of monomer by wild type N. gonorrhoeae is capable of acting in trans to enhance mutant. In contrast, the predominance of FA1090 over FA1090 ΔltgA/ΔltgD would reflect an intrinsic survival defect in the mutant. For each mouse on each day that were positive for N. gonorrhoeae, the competitive index (CI) was calculated. The CI is defined as follows:

\[
CI = \frac{\left( \frac{\text{CFU/mL}}{\text{FA1090 CFU/mL}} \right)_{\text{recovered}}}{\left( \frac{\text{CFU/mL}}{\text{FA1090 CFU/mL}} \right)_{\text{innoculum}}}
\]

The log(CI) reflects the relative fitness of FA1090 and FA1090 ΔltgA/ΔltgD, where log(CI) = 0 reflects an equal fitness and log(CI) > 0 or log(CI) < 0 reflects a fitness defect in FA1090 or in FA1090 ΔltgA/ΔltgD respectively. Over the course of a 10 day infection, the log(CI) was significantly below 0 on each day, except on days where the dynamic range of the assay was
reduced as a result of low bacterial recovery (Figure 3.4A and 3.4B). Individual mice are noted by different symbols and/or symbol color. Mice not appearing as a data point on a single day were the result of no recovery of *N. gonorrhoeae* on that day. The data show that FA1090 ΔltgA/ΔltgD has a fitness defect *in vivo* that was not recovered by the presence of 1,6-anhydro PGN monomers produced during co-infection by FA1090. Similarly, the survival of FA1090 *in vivo* was not affected by the presence of multimeric PGN.

**3.4.5 NOD2 does not play a role in *N. gonorrhoeae* pathogenesis in the murine model of infection**

Given that *N. gonorrhoeae* PGN release is known to cause inflammatory cytokine production by host cells in cell culture, we additionally sought to study the role of NOD2 in *N. gonorrhoeae* pathogenesis. To test the role of NOD2 in the pathogenesis of *N. gonorrhoeae* infection, we infected *Nod2*−/− and wild type mice with FA1090. *N. gonorrhoeae* was collected from mice and quantified by replica plating on GC-VNTCS agar as previously described. *Nod2*−/− mice had the same bacterial burden and persistence of infection as wild type time mice over the course of a 10 day infection (Figure 3.5A-C).

**3.5 Discussion**

The role of PGN monomer production and release by *N. gonorrhoeae* in pathogenesis is not fully elucidated. PGN monomers from both *N. gonorrhoeae* and *Bordetella pertussis* are well-documented to cause death of fallopian tube epithelial cells and ciliated cells of the tracheal epithelium, respectively (Goldman et al., 1982; Melly et al., 1984). Here we show that the lytic transglycosylase proteins LtgA and LtgD responsible for producing these anhydro PGN monomers are important in *N. gonorrhoeae* colonization of the female mouse genital tract. *N. gonorrhoeae* that have the *ltgA* and *ltgD* genes deleted have a fitness defect *in vivo* and are poor
colonizers of the infection site. Whether or not this is a direct consequence of PGN multimer release, failure of monomer release, or the result of yet-to-be characterized effects of \textit{ltg} deletion, such as effects on cell wall integrity or PGN recycling in vivo, is unknown.

We also studied the role of NOD2 in \textit{N. gonorrhoeae} pathogenesis using \textit{Nod2}\textsuperscript{-/-} mice. Somewhat surprisingly, we found that \textit{Nod2}\textsuperscript{-/-} mice had a similar bacterial burden and persistence of infection as wild type mice. One possible explanation for this is the possibility that \textit{N. gonorrhoeae}, an obligate human pathogen, has been fine-tuned to the immune signaling of its human host. Mice are not naturally susceptible to infection with \textit{N. gonorrhoeae} and only become susceptible under administration of 17β-estradiol as well as antibiotics to reduce commensal colonization. Differences in ligand specificity between mouse NOD1 and human NOD1 have been demonstrated. Mouse NOD1, for example, is able to recognize 1,6-anhydro tetrapeptide PGN monomer, while human NOD1 cannot (Magalhaes et al., 2005). During growth, about 20% of the 1,6-anhydro PGN monomers released are the tetrapeptide monomer, while most of the remaining are 1,6-anhydro tripeptide PGN monomer (Sinha and Rosenthal, 1980). Ligand recognition differences between mouse NOD2 and human NOD2, particularly in the context of recognizing 1,6-anhydro PGN fragments, has not been studied. Such differences could manifest in an \textit{in vivo} mouse model infection as compensatory or alternate critical immune signaling for control of infection that are not relevant in human infection. Examples of this have been demonstrated \textit{in vitro}, where NOD2 was shown to be important in controlling \textit{Mycobacterium tuberculosis} infection in human, but not mouse, macrophages (Brooks et al., 2011). Thus, the lack of a phenotype in \textit{Nod2}\textsuperscript{-/-} mice in \textit{N. gonorrhoeae} infection is not a definitive indication that NOD2 does not play a role in infection in humans.
An alternative explanation may be supported by our previous findings, reported in Chapter 2, which show that 1,6-anhydro PGN monomers released by *N. gonorrhoeae* are poorly activating of NOD2. The role of NOD2 in detection of *N. gonorrhoeae* has only recently been reported on, and its function has not been tested *in vivo* through use of a mouse model. Culture supernatants from growing *N. gonorrhoeae* are poor activators of NOD2 (Mavrogiorgos et al., 2013). Our data showing no role for NOD2 in either controlling bacterial burden or infection persistence *in vivo* may confirm *in vitro* findings that wild type *N. gonorrhoeae* is a poor activator of NOD2. An interesting question then arises regarding the role of NOD2 in *N. gonorrhoeae* infection. Given that NOD2 has been shown to play a role in many innate and adaptive immune responses—including autophagy, antimicrobial peptide production, and antigen presentation—avoiding NOD2 activation may be beneficial to survival in the host (Cooney et al., 2009; Travassos et al., 2009; Voss et al., 2006). Since we have observed *in vitro* that FA1090 ΔltgA/ΔltgD causes increased production of inflammatory cytokines and is more activating of NOD2, infection of *Nod2*−/− mice with FA1090 ΔltgA/ΔltgD might better demonstrate the interplay between *N. gonorrhoeae* and NOD2. If NOD2 signaling is responsible for the poor fitness of FA1090 ΔltgA/ΔltgD *in vivo*, then *Nod2*−/− mice infected FA1090 ΔltgA/ΔltgD would have elevated bacterial burdens compared to wild type mice.

FA1090 ΔltgA/ΔltgD does not appear to be more inflammatory *in vivo*, in contrast to *in vitro* data showing that mouse BMDCs produce more IL-1β and TNF-α in response to culture supernatants from FA1090 ΔltgA/ΔltgD when compared to cytokine production in response to wild type FA1090. Our *in vivo* characterization is limited to neutrophil influx, however, and may not sufficiently quantify the full extent of local inflammation. It may explain, however, why FA1090 ΔltgA/ΔltgD is able to survive in the mouse host for a similar length of times as
FA1090. Ultimately, the bacterial burdens in mice infected with FA1090 ΔltgA/ΔltgD may be too low to trigger sufficient PMN migration, and this may explain the higher percentage of PMNs in FA1090 infected mice. Additional experiments are needed in order to make a conclusion regarding the correlation between bacterial burden and PMN influx. There are also other markers of inflammation that can be investigated. While the ability to obtain quantifiable levels of local cytokines can be challenging, physical infection of excised mouse genital tract tissues during infection or quantification of mRNA expression for various host inflammatory responses may reveal a more complete understanding of inflammation induced by FA1090 ΔltgA/ΔltgD.

However, the possibly exists that the mechanism that confers a defect in FA1090 ΔltgA/ΔltgD colonization of the mouse genital tract may not be host mediated. Another lytic transglycosylase, LtgC, is required for separation and bacteria with ltgC deleted have an observed growth defect in vitro (Cloud and Dillard, 2004). While deletion of ltgA and ltgD has not been reported to confer a growth defect in vitro (Cloud-Hansen et al., 2008, and Chapter 2 Figure 2.1), a growth defect in FA1090 ΔltgA/ΔltgD may not be observable in liquid culture, which is challenging with *N. gonorrhoeae* as the bacteria undergo significant lysis after less than 24 hours. A minor growth defect may become more significant in a long-term in vivo growth scenario such as the murine model of infection, which may result in the lower bacterial burden and fitness defect. In support of data showing that ltgA and ltgD deletion does not have a growth defect phenotype, in *Vibrio fischeri* deletion of ltgA and ltgD, which have a similar function in PGN monomer production, also does not result in a growth defect or changes in cell morphology (Adin et al., 2009). That considered, there may be other consequences to deletion of ltgA and ltgD, such as integrity of the cell wall, which may reduce resistance to host antimicrobial factors.
Evaluation of survival of FA1090 ΔltgA/ΔltD in the presence of serum, antimicrobial peptides, ROS, or other stressors is needed to determine if loss of resistance to host antimicrobial factors is an explanation for the colonization defects observed in vivo.

Our data demonstrate for the first time a role for LtgA and LtgD in pathogenesis in vivo in the pathogenic bacteria N. gonorrhoeae, and these proteins may be an important target of study in other bacteria where PGN release in an important factor in host-pathogen interaction. Additional research is needed to understand the mechanisms by which the deletion of ltgA and ltgD confer a fitness defect in vivo. These include investigations into the known PGN recognition receptors, including NOD2, through the use of Nod2−/− mice or siRNA knockdown of NOD2 in human cell lines and evaluation of resistance to host antimicrobial factors. Given the variable expression of NOD2 in different cell types, the site of infection may be important. This presents another possible limitation of using the mouse model for these studies, as the infection site in mice is the vagina, whereas the infection site in humans is typically the cervix or the urethra. It is possible that NOD2 signaling not important for acute clearance of bacteria but plays a role in inducing a protective adaptive immune response. In that case, there might be selective pressure to avoid NOD2 signaling even if there is no benefit in acute infection. As an alternate to studies involving NOD2, our previous in vitro findings show that FA1090 ΔltgA/ΔltD also increases TLR2 activation, which may mediate the reduced survival of the mutant in vivo.
Figure 3.1. Mice infected with FA1090 ΔltgA/ΔltgD have a lower bacterial burden compared to mice infected with wild type N. gonorrhoeae. BALB/c mice were infected with either FA1090 or FA1090 ΔltgA/ΔltgD (JD). Bacterial burden was monitored daily with vaginal swabs. A) Amount of N. gonorrhoeae recovered from mice on each day. Graph shows data for mice known to be colonized with N. gonorrhoeae. A value of 1/2 lower limit of detection (LLD) was used when N. gonorrhoeae was not recovered from a mouse on one day but was recovered on a subsequent day. B) Area under the curve (AUC) of bacterial burden for each mouse over the course of the experiment. Data shown are a combination of two separate experiments. Data were analyzed using GraphPad Prism 6. AUC data was analyzed by Student’s t-test. * = P < 0.05.
Figure 3.2. Deletion of \textit{ltgA} and \textit{ltgD} does not affect infection persistence of \textit{N. gonorrhoeae} in the mouse model.

Mice were infected with either FA1090 or FA1090 \textit{ΔltgA/ΔltgD} and the persistence of infection was monitored over the course of ten days using vaginal swabs. Data shown were the combination of two independent experiments. All data was analyzed using GraphPad Prism 6. Infection persistence data was analyzed using a logrank test. ns = not significant.
Figure 3.3. Increased inflammatory responses to FA1090 ΔltgA/ΔltgD in vitro do not replicate in vivo.
Primary mouse bone marrow derived dendritic cells (BMDCs) were exposed to culture supernatants from FA1090 and FA1090 ΔltgA/ΔltgD and assayed for production of A) IL-1β and TNF-α. Mice infected with FA1090 or FA1090 ΔltgA/ΔltgD were assayed for vaginal neutrophil influx (B). Data from days five, six, and seven of mice for which *N. gonorrhoeae* was recovered (+Ng) or not recovered (-Ng) during the course of the experiment are shown. Data are shown as the mean percent of neutrophils in all mice. The percent of neutrophils for an individual mouse is the mean number of neutrophils relative to other cells types calculated from at least three fields with a total of at least 100 cells. Cytokine data are representative of at least three independent experiments with at least three independent preparations of culture supernatant. *In vivo* data are the combination of two independent experiments (see Appendix 3). Data were analyzed by GraphPad Prism 6. Error bars represent +/- S.E.M. Significance was determined using Student’s T-test, * = P < 0.05.
Figure 3.4. Deletion of ltgA and ltgD in *N. gonorrhoeae* confers a fitness defect in vivo.

A competitive co-infection with FA1090 and FA1090 ΔltgA/ΔltgD was used to evaluate the relative fitness of FA1090 ΔltgA/ΔltgD. Bacterial loads of each were calculated from vaginal swabs taken daily. Competitive index (CI) is defined as FA1090 ΔltgA/ΔltgD recovered/FA1090 recovered and is normalized for differences in the inoculum. A) Log(CI) for each mouse on each day. Data are only shown for mice in which *N. gonorrhoeae* was recovered on that day. Each mouse in the study is represented by different symbols and/or symbol colors. B) Data showing average total recovered *N. gonorrhoeae* (FA1090 + FA1090 ΔltgA/ΔltgD). Mice in which no *N. gonorrhoeae* was recovered are excluded. Data shown is the combination of two independent experiments. Data were analyzed using GraphPad Prism 6. CI data was assessed for being significantly different from 0. Data were confirmed to be normally distributed using a Shapiro-Wilk normality test and significance was determined on each day using a one sample t-test, * = P < 0.05. LLD: lower limit of detection.
Figure 3.5. NOD2 does not play a role in controlling *N. gonorrhoeae* infection.
BALB/c WT or Nod2−/− mice were infected with *N. gonorrhoeae* FA1090. Daily vaginal swabs were used to monitor bacterial burden over the course of ten days. Neither bacterial burden nor infection persistence was altered in Nod2−/− mice. A) Amount of *N. gonorrhoeae* recovered from mice on each day. Graph shows data for mice known to be colonized with *N. gonorrhoeae*. A value of ½ lower limit of detection (LLD) was used when *N. gonorrhoeae* was not recovered from a mouse on one day but was recovered on a subsequent day. B) Area under the curve (AUC) of bacterial burden for each mouse over the course of the experiment. C) Persistence of *N. gonorrhoeae* infection. Data shown is the combination of two independent experiments. All data were analyzed using GraphPad Prism 6. AUC data were analyzed by Student’s t-test. Infection persistence data were analyzed using the logrank test. ns = not significant.
Chapter 4
Discussion and Future Directions

4.1 Discussion

The precise role of peptidoglycan monomer release and NOD2 signaling in the pathogenesis of *N. gonorrhoeae* infection has not been well studied. NOD2 is involved in the induction of many host responses that have been shown to be suppressed during *N. gonorrhoeae* infection or that *N. gonorrhoeae* is resistant to. For example, NOD2 is known to mediate MHC class II antigen-specific CD4+ responses and facilitate polarization of Th2 cells in response to those antigens (Cooney et al., 2009; Magalhaes et al., 2008). In the mouse model of *N. gonorrhoeae*, induction of Th2 responses enhance rate of bacterial clearance (Liu and Russell, 2011; Liu et al., 2013). Th2 responses to infection are suppressed by *N. gonorrhoeae*-induced TGF-β secretion, which in the context of other inflammatory cytokines leads to the predominance of Th17 responses (Liu et al., 2012). Activation of NOD2 by MDP has been shown to stimulate production of antimicrobial peptides like HNP1 and β-defensin-2 (Voss et al., 2006; Yamamoto-Furusno et al., 2010). *N. gonorrhoeae* is able to survive intracellularly even in cells capable of producing antimicrobial peptides, and is resistant to various host antimicrobial agents, including bile salts, progesterone, and antimicrobial peptides (Criss et al., 2009; Hagman et al., 1995; Jerse et al., 2003; Shafer et al., 1998). Nevertheless, while our studies implicate reduced NOD2 signaling as a mechanism of action of the lytic transglycosylases LtgA and LtgD in vitro, *N. gonorrhoeae* infection in *Nod2*−/− mice progressed similarly to infection in wild type mice. It is possible that mouse recognition of PGN through NOD1, NOD2, or other signaling
pathways is sufficiently different than PGN signaling in humans to explain this discrepancy. There is evidence for ligand specificity differences between mouse and human NOD1, as mouse but not human NOD1 can recognize tetrapeptide 1,6-anhydro PGN monomer known as tracheal cytotoxin that is released from *Bordetella pertussis* (Magalhaes et al., 2005). NOD2 is able to control *Mycobacterium tuberculosis* infection *in vitro* in human macrophages but not in mouse macrophages (Brooks et al., 2011). Differences between mouse and human NOD2 with regards to 1,6-anhydro PGN recognition and activation have not been studied. It seems unlikely that *N. gonorrhoeae* would release sufficient quantities of monomeric PGN as to cause epithelial cell damage while also minimizing NOD2 recognition of these fragments if NOD2 was not relevant to pathogenesis. The 1,6-anhydro bond that interferes with human NOD2 signaling does not play a role in inducing ciliated cell death; only a portion of the peptide chain, lactyl-D-glutamic acid-*meso*-diaminopimelic acid, is required for the toxic function of PGN monomers (Luker et al., 1995). In the context of our own *in vitro* data and data published by another group, *N. gonorrhoeae* culture supernatants are poorly activating of NOD2 (Mavrogiorgos et al., 2013). Our data showing that *Nod2*−/− mice maintain similar bacterial burdens and persistence of infection as wild type mice may reflect that *in vivo* *N. gonorrhoeae* minimally activates NOD2 during infection. Culture supernatants from the lytic transglycosylase deletion mutant FA1090 ΔltgA/ΔltgD is a superior activator of human NOD2 *in vitro* relative to wild type FA1090, likely due to the presence of large, multimeric PGN complexes (Chapter 2, Section 2.X). Infection of *Nod2*−/− mice with the mutant strain may reveal a function of NOD2 in infection and the survival benefit for evasion of NOD2 activation. Such studies, along with those described in Chapter 4, Section 4.2, are required to further elucidate a possible role of NOD2 in *N. gonorrhoeae* pathogenesis.
Despite the lack of involvement of mouse NOD2 in controlling infection with *N. gonorrhoeae*, the LtgA and LtgD proteins responsible for the production of released 1,6-anhydro PGN monomers do demonstrate a role in pathogenesis in the mouse model of infection and inflammatory response *in vitro*. *In vitro* LtgA and LtgD reduce inflammatory signaling, as exposure to live FA1090 ΔltgA/ΔltgD or culture supernatants induces the production of significantly more IL-1β and TNF-α compared to wild type FA1090 in a variety of cell types, including THP1 cells, human primary blood dendritic cells, and mouse bone marrow derived dendritic cells. This is likely partially due to an increase in NOD2 signaling by releasing PGN multimers rather than PGN monomers with a 1,6-anhydro bond addition on the muramic acid sugar. We show that 1,6-anhydro PGN monomers are poor activators of NOD2. In contrast reducing forms of PGN, produced through the action of host lysozyme on multimeric PGN, are in contrast highly activating of NOD2. The lack of a significant increase in NOD1, TLR4, and TLR9 activation in response to culture supernatants from FA1090 ΔltgA/ΔltgD suggests the increased NOD2 response is not simply the result of higher levels of bacterial compounds—DNA, LPS, or NOD1-activating PGN—but is rather specific to an increase quantity of NOD2-activating PGN, presumably multimeric PGN that is subsequently being digested by host lysozyme. The finding that the addition of the 1,6-anhydro bond on the muramic acid sugar of PGN monomers blocks NOD2 activation has not been previously reported and is an important addition to the literature in understanding innate immune recognition of bacteria.

Interestingly, we did observe an increase in TLR2 activation in response to culture supernatants from FA1090 ΔltgA/ΔltgD. The role of TLR2 in PGN signaling has been disputed in the literature. PGN was originally identified as a TLR2 ligand, but subsequent studies that used rigorous purification methods on bacterial-derived PGN showed no activation of TLR2,
suggesting that contaminates such as teichoic acid or PGN-associated lipoproteins were activating TLR2 (Schwandner et al., 1999; Takeuchi et al., 1999b; Travassos et al., 2004; Yoshimura et al., 1999). However, a response paper to that study used similar stringent purification methods on *Staphylococcus aureus*-derived PGN showed that multimeric *S. aureus* PGN was an activator of TLR2 (Dziarski and Gupta, 2005). Additional studies have since demonstrated a role of TLR2 in recognition of *S. aureus* PGN through co-localization with NOD2, and that multimeric PGN from Δlgt *S. aureus* mutants, which lack lapidated prelipoproteins, is not recognized by TLR2 alone (Müller-Anstett et al., 2010; Volz et al., 2010). Our results do not show a significant difference in the activation of TLR2 between multimeric and monomeric PGN, and both PGN species only weakly activate TLR2. The increased capacity to induce TLR2 activation by culture supernatants from FA1090 ΔltaΔltgD is likely due to increased quantities of known TLR2 stimulating proteins from *N. gonorrhoeae*, PorB and Lip (Fisette et al., 2003; Massari et al., 2002). Release of these TLR2-activating polypeptides may accompany the release of larger PGN multimers that are not processed into 1,6-anhydro-MurNAc-containing PGN monomers in FA1090 ΔltaΔltgD.

LtgA and LtgD appear to have a distinct role in pathogenesis based on in vivo data from the mouse model of infection. There is a significantly lower bacterial burden in mice infected with FA1090 ΔltaΔltgD compared to mice infected with FA1090. Further, FA1090 ΔltaΔltgD has a clear fitness defect, as demonstrated by competitive infection with mice. Despite this, there was no observable difference in the persistence of infection in the mutant compared to the wild type, though there may be a difference in the early establishment of infection by FA1090 ΔltaΔltgD. This could be partially explained by the low number of local neutrophils observed in response to FA1090 ΔltaΔltgD compared to FA1090. In vitro,
FA1090 ΔltgA/ΔltgD induces the production of more IL-1β and TNF-α compared to FA1090. The discrepancy between the *in vitro* and *in vivo* data may be explained by the low bacterial burden characteristically observed in mice infected with FA1090 ΔltgA/ΔltgD. Bacterial numbers of FA1090 ΔltgA/ΔltgD may not be high enough to induce significant inflammation, and are therefore able to persist for a similar amount of time as FA1090.

Lytic transglycosylases have been implicated in other bacteria as being important to virulence, but in those contexts the lytic transglycosylases play a role in cell separation or cell wall dynamics that are thought to be important in interaction with host cells (Bartoleschi et al., 2002; Stapleton et al., 2007). An example more relevant to the action of lytic transglycosylases in PGN release involves *Vibrio fischeri*, an organism whose PGN monomer release is important in its symbiotic relationship with the Hawaiian bobtail squid (Koropatnick, 2004). A ΔltgA/ΔltgD mutant of *V. fischeri*, which, similar to ΔltgA/ΔltgD *N. gonorrhoeae* produces significantly less PGN monomer, did not have a colonization or fitness defect when tested *in vivo* in squid (Adin et al., 2009). Our data are the first to report on the possible colonization defects in pathogenic bacteria that occur as a result of deletion of the lytic transglycosylases responsible for PGN monomer production.

Our current data do not provide a mechanistic explanation for the reduced fitness of FA1090 ΔltgA/ΔltgD in the mouse model. Though mouse NOD2 does not appear important in infection with wild type *N. gonorrhoeae*, this does not exclude the possibility of a role for NOD2 in response to FA1090 ΔltgA/ΔltgD. Gonococci may be more susceptible to killing by host phagosomes due to the increased production of NOD2-activating PGN. Subsequent pathways downstream of NOD2, such as autophagy or the induction of host antimicrobial peptides may be detrimental to phagocytosed organisms. Comparing the bacterial burden of FA1090
\(\Delta ltgA/\Delta ltgD\) and FA1090 wild type in a \textit{Nod2}\(^{-}\) mouse could provide an answer to this question. Alternatively, despite lack of \textit{in vitro} evidence that deletion of \textit{ltgA} and \textit{ltgD} cause growth defects, these mutants may a growth defect \textit{in vivo}. These lytic transglycosylase mutants may also have increased susceptibility to host antimicrobial factors.

### 4.2 Future Directions

The studies presented here investigate the role the monomers play in pathogenesis, inflammation, and receptor activation by disrupting the production of 1,6-anhydro peptidoglycan monomers in \textit{N. gonorrhoeae} through the deletion of the genes encoding two lytic transglycosylases, \textit{ltgA} and \textit{ltgD}. This approach has led to the discovery that LtgA and LtgD have a distinct role in suppressing inflammation, through both NOD2 as a result of PGN breakdown and through TLR2 by an unknown mechanism. Additionally, \textit{N. gonorrhoeae} lacking LtgA and LtgD are poor colonizers of the female mouse genital tract and have a clear fitness defect \textit{in vivo}. However, there are some critical limitations in using this approach to understanding the role of PGN monomers in \textit{N. gonorrhoeae} infection. \textit{N. gonorrhoeae} lacking LtgA and LtgD are still releasing large quantities of peptidoglycan. Production and release of these multimeric fragments still expose the host to a NOD1-activating ligand, as we have shown here (Chapter 2, Section 2.X). Additionally, like monomeric PGN, the multimeric fragments contain the peptide chain moiety responsible for epithelial cell damage that occurs during infection (Luker et al., 1995; Melly et al., 1984). Thus, other approaches, such as altering the amount of PGN released by \textit{N. gonorrhoeae}, provide an important extension to the studies on \textit{N. gonorrhoeae} PGN discussed here.

Deletion of the \textit{ampD} or \textit{ampG} in \textit{N. gonorrhoeae} provides such an opportunity. Deletion of \textit{ampD}, whose protein product AmpD is a cytoplasmic N-acetylmuramyl-l-alanine
amidase, results in *N. gonorrhoeae* whose levels of PGN release are significantly reduced (Garcia and Dillard, 2008). A reduction in PGN release can also be achieved by replacing the gonococcal *ampG*, a permease, with meningococcal *ampG*, resulting in a reduction of PGN release from 15% to 4%, an amount similar to that observed in other gram-negative bacteria such as *Escherichia coli* (Woodhams et al., 2013). This occurs as a result of meningococcal *ampG* being more efficient at recycling PGN than gonococcal *ampG*. A similar strategy was used in the construction of an attenuated *Bordatella pertussis* strain for vaccine use, where *B. pertussis ampG* was replaced with *E. coli ampG* to reduce the amount of toxic PGN monomers released (Mielcarek et al., 2006). An increase in PGN monomer release can also be achieved: deletion of *ampG* in *N. gonorrhoeae* causes a substantial (7-fold) increase in the amount of PGN released (Garcia and Dillard, 2008). Both the *ampG* and *ampD* deletion mutants are reported to have similar *in vitro* growth characteristics to the isogenic wild type strain, and thus in that respect are suitable for *in vitro* and *in vivo* studies using live *N. gonorrhoeae*. Identification of a phenotype for *ampG* or *ampD* mutant *N. gonorrhoeae* in *vivo* using the mouse model of infection in comparative and competitive infections with the wild type strain could provide further insights into the role of PGN *in vivo*. Use of these strains could also open up avenues of *in vitro* studies on PGN release including recognition and killing of *N. gonorrhoeae* by neutrophils or infiltrating APCs. Because ΔltgA/ΔltgD *N. gonorrhoeae* appear to produce equal amounts of NOD1-stimulating ligand during growth as wild type (Chapter 2, Section 2.X), the use of ΔampD *N. gonorrhoeae* would reduce the total amount of released PGN and mechanistic roles for both NOD1 and NOD2 in triggering an immune response could be better explored. The same follows for use of ΔampG, which would increase the amount of NOD1 and NOD2-activating PGN, the latter of which is notably present in only minimal quantities, due to the majority of released PGN.
being the 1,6-anhydro monomer, which does not activate NOD2. An ampG mutant of *Shigella flexneri* that released more PGN than wild type was found to increase NOD1, but not NOD2 activation (Nigro et al., 2008).

There are reported differences between the ligand specificities and role in controlling the immune response to bacterial infection between human and mouse NOD1 and NOD2 that may represent a clear limitation of studying *N. gonorrhoeae* PGN in a mouse infection model and using mouse-derived cells. Mouse NOD1 can recognize tetrapeptide PGN monomer, while human NOD1 cannot (Magalhaes et al., 2005). Differences in NOD2 signaling between mouse and human concerning the anhydro PGN fragments discussed in Chapter 2 have not been reported and such an experiment may provide insight into the relevancy of the mouse model of *N. gonorrhoeae* infection. Human NOD2 has been shown to play a role in the control of *Mycobacterium tuberculosis* in macrophages *in vitro* while mouse NOD2 does not (Brooks et al., 2011). Thus, even if mouse NOD2 has similar limitation in detecting PGN fragments that contain a 1,6-anhydro bond, there may be critical differences in downstream signaling.

Given these potential problems with the mouse model of infection discussed above, use of the human model of infection in men to study the role of monomeric PGN release in *N. gonorrhoeae* infection may provide the only relevant data for this scientific question. The use of this model presents its own scientific challenges—low numbers of volunteers, studies are limited to infection of the male urethra, for example—but a competitive co-infection with FA1090 and FA1090 ΔltgA/ΔltgD (or FA1090 ΔampD or ΔampD if preliminary studies prove sufficiently interesting) would reveal the role of LtgA and LtgD in the fitness of this bacteria in humans. *In vitro* studies making use of siRNA to knockdown NOD2 or NOD1 in human primary or
immortalized cells could provide some mechanistic explanations of an observed *in vivo* phenotype.

There is still a significant amount of work to be done in understanding the role of *N. gonorrhoeae* PGN and PGN receptors—including NOD1 and NOD2—in infection. Undoubtedly additional proteins that function to regulate the production and release of monomeric PGN will be discovered in the future. Currently the mechanism by which PGN monomers are transported, actively or passively, across the cell membrane is unknown. Further characterization, both *in vivo* and mechanistically, of *N. gonorrhoeae* mutants that disrupt wild type monomeric PGN release are needed to add to the understanding of how *N. gonorrhoeae* interacts with the host immune system and suppresses both innate and protective adaptive immune responses.
APPENDIX 1: CONSTRUCTION OF FA1090 ΔLTGA/ΔLTGD (KK)

Alternate construction of FA1090 ΔltgA/ΔltgD

*Neisseria gonorrhoeae* strain FA1090 ΔltgD was generated as described previously (Cloud and Dillard, 2002; Cloud-Hansen et al., 2008) and deletion was confirmed by PCR. Construction of FA1090 ΔltgA/ΔltgD (KK) was done in FA1090 ΔltgD using the *ermC/rpsl* ltgA insert plasmid described previously by spot transformation on GC agar (Johnston and Cannon, 1999). Erythromycin-resistant colonies were chosen, and insertion of the plasmid was confirmed by PCR. However, a PCR product of the repair plasmid insertion was used to retransform the mutant, rather than using the repair plasmid as described. Transformants were selected on streptomycin and were confirmed to have erythromycin susceptibility. Clean deletion of *ltgA* was confirmed by PCR, shown below, and the sequence was verified.
APPENDIX 2: FA1090 ΔLTGA/ΔLTGD (KK) IN VITRO DATA

FA1090 ΔltgA/ΔltgD (KK) has a similar in vitro phenotype as FA1090 ΔltgA/ΔltgD (JD).

As seen with FA1090 ΔltgA/ΔltgD (JD), FA1090 ΔltgA/ΔltgD (KK) induces the production of more inflammatory cytokines IL-1β and TNF-α in comparison to wild type FA1090 in human derived cell lines. This was reproducible with both PMA-stimulated THP1 cells exposed to live N. gonorrhoeae (MOI 0.1) and THP1 cells exposed to N. gonorrhoeae culture supernatants, which were generated as described in Chapter 2. Similar phenotypes are also observed in transfected immune receptor-expressing cells. FA1090 ΔltgA/ΔltgD (KK) was more activating of both NOD2 and TLR2, but not NOD1.
FA1090 ΔltgA/ΔltgD (KK) produces similar inflammatory and receptor activation responses as FA1090 ΔltgA/ΔltgD (JD)

A) PMA-treated THP1 cells were exposed to live *N. gonorrhoeae* (multiplicity of infection: 0.1) and production of IL-1β and TNF-α in the cell culture supernatant was measured by ELISA. Data are representative of two independent experiments. B) THP1 cells were exposed to *N. gonorrhoeae* culture supernatants and production of IL-1β and TNF-α was measured by ELISA. HEK293 cells transfected with a secreted alkaline phosphatase reporter and C) NOD1, D)
NOD2, or E) TLR2 were exposed to *N. gonorrhoeae* culture supernatant. Data are expressed as fold activation over basal levels of activation in response to graver wade media controls. For experiments using *N. gonorrhoeae* culture supernatants, data is representative of at least two independent experiments with at least two preparations of culture supernatants. Data were analyzed using GraphPad Prism 6 using Student’s t-test, * = P < 0.05.
APPENDIX 3: FA1090 ΔLtgA/ΔLtgD (KK) IN VIVO DATA

FA1090 ΔltgA/ΔltgD (KK) has a similar in vivo phenotype as FA1090 ΔltgA/ΔltgD (JD).

BALB/c mice were infected with either FA1090 or FA1090 ΔltgA/ΔltgD (KK) and bacterial burden was monitored daily with vaginal swabs as described in Chapter 3. Notably, bacterial burdens for both wild type FA1090 and FA1090 ΔltgA/ΔltgD (KK) were lower than is typically observed in these experiments. In addition, an unusually low number of mice were successfully infected with *N. gonorrhoeae*, resulting in a low number of subjects for analysis. The bacterial burden of FA1090 ΔltgA/ΔltgD (KK) is trending lower than that of FA1090, though the difference in area under the curve (AUC) between the mutant and wild type is not statistically significant (Panel A and B, P = 0.1027). As observed with FA1090 ΔltgA/ΔltgD (JD), infection persistence was similar between FA1090 and FA1090 ΔltgA/ΔltgD (KK) (Panel C). Additional experiments are needed to increase the number of animals in each group and confirm these findings and phenotype similarity to FA1090 ΔltgA/ΔltgD (JD).
Preliminary data shows that FA1090 ΔltgA/ΔltgD (KK) has a similar *in vivo* phenotype as FA1090 ΔltgA/ΔltgD (JD).

A) Amount of *N. gonorrhoeae* recovered from mice on each day. Graph shows data for mice known to be colonized with *N. gonorrhoeae*. A value of ½ lower limit of detection (LLD) was used when *N. gonorrhoeae* was not recovered from a mouse on one day but was recovered on a subsequent day. B) Area under the curve (AUC) of bacterial burden for each mouse over the course of the experiment. C) Persistence of infection. Data shown are a combination of two separate experiments. Data were analyzed using GraphPad Prism 6. AUC data was analyzed by Student’s t-test.
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