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***Neisseria gonorrhoeae* lytic transglycosylases LtgA and LtgD reduce host innate immune signaling through TLR2 and NOD2**

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Abstract

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-1 β and TNF- α . Culture supernatants from *ltgA*/*ltgD* *N. gonorrhoeae* contain more shed outer membrane-associated proteins and multimeric peptidoglycan fragments rather than monomers. These culture supernatants were more potent activators of host TLR2 and 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 reduce recognition of *N. gonorrhoeae* by TLR2 and NOD2.

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Author Contributions

K.J.K. designed and performed experiments and wrote the manuscript. K.T.H., J.E.A., and C.W. generated critical reagents and performed experiments. J.P.D. and J.A.D. assisted in the conceptualization and design of experiments and edited the manuscript.

Keywords

NOD2; peptidoglycan; lytic transglycosylase; lysozyme; *Neisseria gonorrhoeae*; innate immunity

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 peptidoglycan¹. 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 tissues². 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%)³. 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 airways². Monomeric PGN fragments from *N. gonorrhoeae* were later shown to induce damage to the mucosa of cultured human fallopian tubes⁴. The *N. gonorrhoeae* genome encodes seven lytic transglycosylases capable of liberating PGN from *N. gonorrhoeae* sacculi, but only LtgA and LtgD are responsible for the production of 1,6-anhydro-MurNAc-containing PGN monomers released by *N. gonorrhoeae* during growth in culture⁵. Instead of releasing monomeric 1,6-anhydro-

MurNAc-containing PGN monomers, *N. gonorrhoeae* lacking both *ltgA* and *ltgD* release a variety of multimeric PGN fragments⁵.

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 NF- κ B and subsequent inflammatory cytokine production in response to the PGN components D-glutamyl-*meso*-diaminopimelic acid (iE-DAP) or muramyl dipeptide (MDP) depend on host NOD1 and NOD2, respectively^{6–9}. Recent studies have demonstrated that iE-DAP and MDP are direct ligands for NOD1 and NOD2, respectively^{10–12}. 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 itself¹³. Recognition of bacterial PGN allows the host to initiate antimicrobial responses^{14,15}. The role PGN monomer production by *LtgA* and *LtgD* plays in the host response to *N. gonorrhoeae* infection has not been elucidated.

In this study, we sought to assess the role of *N. gonorrhoeae* *LtgA* and *LtgD*-mediated PGN monomer release on the innate immune response and the specific immune receptor responses to *N. gonorrhoeae* PGN monomers. We found 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, reducing 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 inflammatory cytokine signaling by impacting at least two separate innate immune signaling pathways, TLR2 and NOD2.

Results and Discussion

To assess the effect of *N. gonorrhoeae* *LtgA*- and *LtgD*-mediated peptidoglycan release on host monocyte derived cell cytokine production, monocyte-derived THP1 cells differentiated towards a macrophage-like phenotype by treatment with phorbol-myristic acid were exposed to wild type *N. gonorrhoeae* strain FA1090 or an isogenic mutant with deletions of the *ltgA* and *ltgD* genes (FA1090 *ltgA* *ltgD*) and the accumulation of IL-1 β and TNF- α in the cell culture supernatants was measured¹⁶. Cells exposed to FA1090 *ltgA* *ltgD* secreted significantly greater quantities of both cytokines than those exposed to the isogenic parent (Figure 1A). Culture supernatants from FA1090 *ltgA* *ltgD* also elicited greater inflammatory cytokine production from THP1 cells and primary human dendritic cells compared to culture supernatant from the parental strain (Figure 1B and 1C). The enhanced TNF- α production in response to *N. gonorrhoeae* conditioned media caused by deletion of *ltgA* and *ltgD* was reversed by expression of both enzymes from an exogenous site but not by expression of either alone (Figure 1D). 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 *ltgA/ltgD* FA1090 strains, bacterial growth of each strain was assessed over a four-hour growth period by monitoring the optical density of the cultures and no significant difference in the optical density was observed between strains (Figure 1E). Size exclusion chromatography of culture supernatant after metabolic labeling of the bacteria with [6-³H]glucosamine confirmed that *ltgA/ltgD* FA1090 released dimeric and multimeric PGN while wild type FA1090 released primarily monomeric PGN with a small peak of dimeric PGN (Figure 1F), as previously described for *N. gonorrhoeae* strain MS11⁵. Overall, these data demonstrate that culture supernatants containing multimeric PGN fragments released from *ltgA/ltgD N. gonorrhoeae* have an enhanced capacity to elicit host inflammatory cytokine production.

To identify immune receptors involved in increased host cytokine response to FA1090 *ltgA/ltgD*, we used commercially available HEK293 reporter cells stably transfected with an NF- κ B-responsive secreted alkaline phosphatase reporter gene and NOD1, NOD2, TLR2, TLR4, or TLR9. FA1090 *ltgA/ltgD* culture supernatants consistently induced greater NOD2 activation, 1.5-fold increased secreted alkaline phosphatase activity versus 1.1-fold activation over levels generated by untreated and Graver-Wade Medium-treated cells (Figure 2A). Similarly, culture supernatants from FA1090 *ltgA/ltgD* 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 2B). In contrast, FA1090 and FA1090 *ltgA/ltgD* culture supernatants induced similar reporter activation to one another when applied to NOD1-, TLR4-, (Figure 2C and 2D) and TLR9-expressing cells (Data not shown). HEK293 cells carrying only the reporter without expression of any exogenous innate immune receptors demonstrated no significant reporter activation when treated with FA1090 or FA1090 *ltgA/ltgD* culture supernatants, though they did respond to stimulation with the cytokine TNF-alpha (Figure 2E). Some studies have implicated TLR2 in the host response to PGN while others studies suggest that TLR2 has no direct ability to recognize PGN. It has been proposed that TLR2 responds to multimeric PGN but not monomeric PGN. Others have proposed that PGN signaling via TLR2 is the result of contaminants, such as lipoproteins or teichoic acids^{13,17,18}. Further, *N. gonorrhoeae* is known to shed outer membrane vesicles that contain lipoproteins, such as Lip, and the protein PorB, which have been shown to activate TLR2^{19,20}. Supernatants from bacterial cultures of equivalent density were assessed by gel electrophoresis and general protein, which demonstrated an increased abundance of protein from the FA1090 *ltgA/ltgD* culture when compared to its isogenic parental strains (Figure 2F). Immunoblot analysis with antibodies directed against the *N. gonorrhoeae* PorB and the lipoprotein Lip, both known to activate TLR2, demonstrated that both proteins were in higher abundance in culture supernatants from FA1090 *ltgA/ltgD* than those from the parental FA1090 strain (Figure 2G)^{19,21}. These data indicate that *ltgA* and *ltgD* reduce the release of gonococcal membrane proteins in addition to being responsible for PGN monomer release from the growing bacteria.

Recently, Mavrogiorgos and colleagues demonstrated that *N. gonorrhoeae* culture supernatant, which contains secreted 1,6-anhydro-MurNAc-containing PGN monomers, robustly activates NOD1, while PGN polymer-containing lysates of whole *N. gonorrhoeae* stimulate both NOD1 and NOD2 equally²². We sought to determine if the PGN content of

the FA1090 *ltgA*/*ltgD* culture supernatants was the causative agent of the increased TLR2 and NOD2 activation. Because culture supernatants from *ltgA*/*ltgD* *N. gonorrhoeae* contain decreased levels of anhydro PGN monomers and increased levels of multimeric PGN (Figure 1E), we sought to test whether polymeric PGN has an enhanced capacity to stimulate NOD2 and TLR2 compared to monomeric PGN. Soluble monomeric and multimeric PGN was generated by complete or partial digestion of *N. gonorrhoeae* sacculi with recombinant LtgA. The quantity of PGN monomeric units in the preparations was assessed by quantitating the free amine group of the *meso*-DAP with an amine-reactive dye. Equivalent quantities of monomeric PGN units, whether in monomeric or multimeric form, were used in innate immune receptor activity assays (Figure 3A). The ability of these isolated monomeric and multimeric PGN to stimulate NOD1, NOD2, and TLR2 using HEK293 reporter cell lines in the absence of other bacterial-derived factors that are found in the conditioned media studied in previous experiments was tested. The secreted alkaline phosphatase activity measured from TLR2-expressing cells stimulated with purified PGN fragments demonstrated no significant difference between stimulation with monomeric and multimeric PGN (Figure 3B). Overall, because isolated gonococcal PGN, in either multimeric or 1,6-anhydro-MurNAc-containing monomeric form, equivalently activated TLR2, LtgA and LtgD most likely modulate host TLR2 signaling by reducing the release of TLR2-activating outer membrane proteins rather than through the alteration of released PGN species. We hypothesize that the release of these TLR2-activating polypeptides accompanies the release of larger PGN multimers that are not processed into 1,6-anhydro-MurNAc-containing PGN monomers in this strain. It is also possible that deletion of *ltgA* and *ltgD* causes an increase in the number of or lipoprotein content of outer-membrane vesicles that are released by *N. gonorrhoeae* during its growth. Recently, Ragland et al demonstrated that the outer-membrane integrity of *N. gonorrhoeae* strain MS11 *ltgA*/*ltgD* was compromised when compared to the parental MS11 strain, which is consistent with our findings of increased release of outer membrane-derived proteins from this strain²³.

Both monomeric 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 3C). Because the cells were stimulated with quantities of DAP estimated to be equivalent, the reason for the difference in NOD1 activation between these two forms of PGN is unclear. It is possible that either multimeric PGN is not taken up as well as monomeric PGN by the HEK293 cell derivatives. It is also possible that binding of NOD1 to multimeric PGN blocks additional iE-DAP-containing motifs within the multimeric PGN structure from binding additional NOD1. Finally, it is also possible that there is quenching or incomplete reaction of DAP with the amine reactive agent used to quantitate these preparations within the multimeric PGN structure. Despite the more robust activation of NOD1 observed with monomeric PGN when compared to multimeric PGN, incubation of the NOD2-expressing cells with monomeric PGN failed to induce activation of reporter gene activity. However, treatment with the PGN multimers that had demonstrated limited activation of NOD1 caused significant activation of NOD2 (3.3-fold induction of SEAP reported activity). We observed that incubation with monomeric PGN did not induce significant activation of NOD2 (1.3-fold induction, $p>0.05$) (Figure 3D). The stimulation

observed in NOD1-, NOD2-, and TLR2-expressing cells was not observed in cells expressing only the reporter construct, indicating that the effects of multimeric and monomeric PGN on reporter gene activation resulted from the transfected receptors and not endogenous innate immune receptors (Figure 3E). To ensure that the reduced NOD2 signaling activation of LtgA-liberated monomeric PGN was not the result of inhibition of NOD2 or blockade of NOD2 ligand uptake in the reported cells, the effects of monomeric PGN on both MDP and multimeric PGN NOD2 stimulation was tested. Monomeric PGN was not found to reduce the NOD2 signaling by either active ligand (Figure 3F), indicating that LtgA was not producing a NOD2 antagonist through its action on *N. gonorrhoeae* PGN.

Because NOD2 can be stimulated by monomeric MDP, the increased NOD2 signaling induced by multimeric PGN was unlikely to be attributable exclusively to the multimeric structure of the PGN. Host cellular lysozyme digestion of cell wall PGN has been previously shown to facilitate NOD2 recognition of *Streptococcus pneumoniae*²⁴. We hypothesized that multimeric PGN released by the *ltgA/ltgD* *N. gonorrhoeae* might stimulate NOD2 effectively if it were first acted on by host lysozyme. To test this hypothesis, we generated PGN monomers from *N. gonorrhoeae* PGN using either recombinant LtgA or human neutrophil lysozyme. Isolated monomeric fragments were tested for activation of NOD1 and NOD2. 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 3G). However, in NOD2-expressing reporter cells, lysozyme-digested PGN monomers were more potent activators of NOD2 signaling when compared to LtgA-digested PGN monomers (Figure 3H).

MurNAc-containing PGN monomers produced by the action of host lysozyme on multimeric PGN differ in chemical structure from monomeric PGN generated by LtgA, which contains a 1,6-anhydro-MurNAc moiety rather than a reducing MurNAc moiety (structures shown in Figure 4A). To test whether the reduced NOD2 signaling by LtgA-generated monomers was the result of the 1,6-anhydro-MurNAc moiety 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 the identity of these isolated monomers was confirmed using mass spectrometry (Figure 5A, B, and C). Each monomeric species was tested using NOD1- or NOD2- expressing reporter cell lines. There was no difference in NOD1 activation between the 1,6-anhydroor 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 5D). 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 μ M) and 3.9-fold (800 nM) increase in NOD2 activation (Figure 5E).

Additionally, 1,6-anhydro-MurNAc-containing PGN tetrapeptide (TCT) failed to activate NOD2, while the reducing-MurNAc-containing PGN tetrapeptide (8 μ M) also activated NOD2, a 8.2-fold increase over basal levels. Mass spectrometry indicated that the purified

PGN monomer preparations did not contain contaminating MDP or dipeptide monomer. 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. The activation of NOD1, but not NOD2, by 1,6-anhydro-MurNAc PGN agrees with previous reports that TCT does not activate human NOD2 but that PGN structures containing iE-DAP in the peptide chain can activate NOD1^{25,26}. Chemical modification of the amine at the 2 position of the muramyl group has been shown to modulate MDP-stimulated NOD2 activation^{27,28}. Our 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.

Our reported findings combined with previous data suggest that *N. gonorrhoeae* maintains multiple mechanisms to suppress host NOD2 signaling by peptidoglycan. Peptidoglycan modification via O-acetylation has previously been shown to increase PGN resistance to lysozyme digestion in many pathogenic bacteria, including *N. gonorrhoeae*^{29–33}. The PGN O-acetyltransferase in *Staphylococcus aureus* has been shown to modulate host cytokine responses to the bacteria by modulation of NOD2 signaling³⁴. Interestingly, while suppressing host NOD2 signaling, *N. gonorrhoeae* does release substantial quantities of NOD1-activating ligand, suggesting the two receptors play non-redundant roles in gonococcal pathogenesis. It is yet to be determined if the ability of LtgA and LtgD to allow *N. gonorrhoeae* to avoid NOD2 signaling or produce PGN fragments that stimulate NOD1 are beneficial to the bacteria during the establishment, persistence, and/or transmission of infection. Indeed, penicillin resistance in *Neisseria meningitidis* was found to lead to changes in the bacterial PGN structure that result in diminished NOD1 signaling and attenuation of the bacteria in animal models of disease³⁵. Additionally, *ltgA/ltgD N. gonorrhoeae* was also recently shown to be more susceptible to killing by human neutrophils than the isogenic parental strain²³. Interestingly, the mutant bacteria were found to be more susceptible to host-derived lysozyme than the parental strain due to the ability of the enzyme to pass through the lower-integrity outer membrane of the mutant strain and access the periplasmic PGN cell wall. Consistent with our findings of enhanced cytokine secretion caused by the *ltgA/ltgD* mutant *N. gonorrhoeae*, Ragland *et al* also noted that *ltgA*- and *ltgD*-deficient *N. gonorrhoeae* caused more robust stimulation of human neutrophils as demonstrated by increased primary and secondary granule exocytosis when compared to stimulation by the parental strain²³. Unfortunately, determining the separate contributions of these two effects of LtgA and LtgD activity on gonococcal infection pathogenesis using the murine model of *N. gonorrhoeae* infection presents a challenge due to the differences between mouse and human NOD1 ligand specificity²⁶.

High levels of NOD2 expression are restricted to phagocytic cells and some specialized epithelial cells, like Paneth cells of the ileal crypts^{36,37}. NOD1 is broadly expressed in epithelial cells and immune cells, though curiously not in neutrophils^{36–38}. This host cell expression restriction of NOD1 may indicate potential mechanisms that have led to selective pressure on *N. gonorrhoeae* to evade host NOD2 signaling. Activation of NOD2 by MDP has been shown to stimulate production of antimicrobial peptides like HNP1 and β -defensin-2^{39,40}. NOD2 also contributes to the development of adaptive immune responses that are weak or absent in humans infected with *N. gonorrhoeae*⁴¹. Suppression of NOD2

activation by *N. gonorrhoeae* LtgA and LtgD may therefore provide a mechanism 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*.

Experimental Procedures

N. gonorrhoeae strains

N. gonorrhoeae strain FA1090 and two isogenic lytic transglycosylase mutant strains, *ltgD* and *ltgA/ltgD* were generated as described previously^{5,42}. Whole genome sequencing of FA1090 *ltgA/ltgD* 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. FA1090 *ltgA/ltgD* strains with complemented expression of LtgA, LtgD, or both were generated by transforming FA1090 *ltgA/ltgD* with plasmid pKH96, pRS62, or both as previously described for complementation of *N. gonorrhoeae* strain MS11 *ltgA/ltgD*²³.

Culture Supernatants

N. gonorrhoeae strains were grown overnight on GCB agar plates, suspended at OD₆₀₀ = 0.2 in 10 mL Graver-Wade media⁴³, 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. Final bacterial densities varied between 5.6×10^6 – 8.3×10^7 for FA1090 and 5.7×10^6 – 6.2×10^7 CFU/ml for FA1090 *ltgA/ltgD*. Culture supernatants were generated by centrifugation at 1200×g for 10 min. followed by filtration through a 0.2 µm filter on at least three separate days.

Analysis of PGN fragment release

Gonococcal strains were metabolically labeled by growth for 45 min in GCB liquid medium lacking glucose and containing 0.4% pyruvate as a carbon source plus 10 µCi/ml [6-³H]-glucosamine (American Radiolabeled Chemicals) to label both the GlcNAc and MurNAc residues in the cell wall. Following the pulse-labeling, cultures were adjusted by dilution to obtain equivalent amounts of radioactive label in the cells for the chase period. Cultures were grown for 2.5 h in GCB liquid medium containing glucose, and the supernatants were collected and filtered through a 0.2 µm filter. PGN fragments in the supernatants were separated by size exclusion chromatography using tandem 350 ml Bio-Gel P6 and Bio-Gel P30 columns eluted with 0.1M LiCl^{44,45}. Radioactivity in the fractions was determined by scintillation counting.

Purification of PGN Fragments

Peptidoglycan fragments from *N. gonorrhoeae* were isolated as described previously⁵. Briefly, PGN sacculi were isolated from *pacA mshB N. gonorrhoeae*. 1,6-anhydro murNAC-containing PGN monomers were obtained by digesting sacculi with soluble LtgA at 37° overnight (monomers) or 15 minutes (multimers). Recombinant, hexahistidine-tagged,

soluble LtgA was expressed in *E. coli* strain BL21(DE3) (Novagen) harboring the expression plasmid pRS95⁴⁶. The recombinant enzyme was subsequently isolated using Immobilized Metal Affinity Chromatography as described by Schaub *et al*⁴⁶. Monomeric PGN containing the reducing sugar was obtained by digesting sacculi with human neutrophil lysozyme (MP Biomedicals). Reactions were stopped by boiling for 10 min, centrifuged to remove insoluble material, and filtered by passage through a 10 kD molecular cut-off Centricon centrifugal filter. Tripeptide and tetrapeptide monomers were purified by reversed phase HPLC using a 5 μ pore size 10-mm by 250-mm Waters XSelect CSH C18 column. Separation was achieved with a 4 to 13% acetonitrile gradient in 0.05% TFA in water at a flow rate of 4.7 mL/min⁴⁷. Peptidoglycan structures were confirmed by mass spectrometry. LC-MS was performed as described by Lenz *et al* with an Agilent 1200 series LC/MSD TOF using electrospray ionization in the positive mode⁴⁸. Peptidoglycan was quantified using a Fluoraldehyde OPA (o-phthalaldehyde) Reagent solution (Thermo Scientific Pierce), which reacts with the single primary amine present on the diaminopimelic acid of a monomeric PGN unit. Standard curves used to estimate the molar concentration of PGN were generated using isoleucine and phenylalanine. Isolated, lyophilized monomeric or multimeric PGN or standards dissolved in water (25 μ L) were added to 25 μ L Fluoraldehyde OPA (o-phthalaldehyde) Reagent in an opaque 384-well microtiter plate. After 1 minute incubation at room temperature, the fluorescence at excitation 350 nm and emission at 450 nm was measured.

Cell culture and cytokine analysis

THP1 cells were grown in suspension in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 50 U/mL penicillin, and 50 μ g/mL streptomycin. Human dendritic cells were generated from peripheral blood obtained from subjects enrolled in a UNC IRB approved study (Study #05-2860) after obtaining informed consent. CD34+ cells were cultured 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 then de-identified prior to transfer to our laboratory for exposure to *N. gonorrhoeae* culture supernatant⁴⁹. The use of these 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)]. 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 medium was used. TNF- α and IL-1 β cytokine analyses on cell supernatants were done using ELISA (BD Biosciences).

Receptor Response

Cells stably expressing human NOD1, NOD2, TLR2, TLR4, or TLR9 and an alkaline phosphatase reporter (HEK-BlueTM, Invivogen) were seeded in 96 well plates with 180 μ L/well of DMEM supplemented with FCS and antibiotics as recommended by the manufacturer and stimulated with *N. gonorrhoeae* culture supernatants (20 μ L/well) or isolated PGN at the concentration indicated overnight. Reporter activity was quantified using a colorimetric assay (Quanti-BlueTM, Invivogen).

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Figure 1.

Deletion of the *ItgA* and *ItgD* genes in *N. gonorrhoeae* results in increased inflammatory signaling in human monocytes and dendritic cells. IL-1 β (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 Graver-Wade Media or supernatant from *N. gonorrhoeae* cultured in Graver-Wade Media as described in the Experimental Procedures (20 μ l per ml of cell culture medium, culture supernatant from bacteria equivalent to an Multiplicity of Infection $\approx$ 0.1). Secreted cytokines were below the level of detection (15.6 pg/ml for IL-1 β and 78 pg/ml for TNF- α) for untreated cells or cells treated with Graver-Wade Media. (D) THP1 cells were exposed to culture supernatants from *N. gonorrhoeae* strain FA1090, FA1090 *ItgA* *ItgD*, and FA1090 *ItgA* *ItgD* transformed with the indicated complementation plasmid and secreted TNF- α was assessed as described above. (E) Optical density of indicated *N. gonorrhoeae* strain cultures at the indicated time points. (F) Size exclusion chromatography profiles of PGN fragments released from FA1090 or FA1090 *ItgA* *ItgD* after labeling of PGN pool with [6- 3 H]-glucosamine. Data points are plotted as mean $\pm$ S.E.M. from three

samples of cells each treated separately with bacteria (A) or cells treated with three preparations of conditioned media generated on three different days (B, C, and D). Plots are representative of repeated experiments (for A–B, n>3 for C–E, n=2). Significance was determined using Student's T-test to compare results from FA1090 to FA1090 *ltgA*/ *ltgD* with the determined p-value indicated. One-way ANOVA with Holm-Sidak's post-hoc test for multiple comparisons was used to compare multiple FA1090-derived strains in (B).

Figure 2.

Culture supernatants from *N. gonorrhoeae* lacking *lta* and *ltb* genes exhibit enhanced activation of human NOD2 and TLR2, but not NOD1. (A) NOD2, (B) TLR2, (C) NOD1, (D) TLR4, and (E) Null HEK293-Blue™ cells were treated with Graver-Wade Media or culture supernatants from the indicated *N. gonorrhoeae* strains (20 μ l culture supernatant added to 180 μ l cell culture medium) 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 Media for each independent experiment. Each cell line was stimulated with a positive control ligand for the

innate immune receptor it expressed: L18-Muramyl Dipeptide (A), Pam3CSK4 (B), iE-DAP (C), and Lipopolysaccharide from *E. coli* K12 (D). Recombinant TNF- α was used to stimulate the Null HEK293-Blue™ which does not overexpress an innate immune receptor (E). (F & G) Equal volumes (15 μ l) from culture supernatants from the indicated strains of *N. gonorrhoeae* were subjected to SDS-PAGE and analyzed by silver staining (F) or immunoblot (G) using antibodies directed against PorB (left) and lipoprotein Lip (right). Data shown represent mean values $\pm$ S.E.M. from cells treated with at least two preparations of culture supernatants and experiments were repeated at least twice. Significance was determined using Student's T-test to compare results from FA1090 to FA1090 *ltgA*/ *ltgD*. A p-value < 0.05 was considered significant.

Figure 3. Multimeric PGN and monomeric PGN released by *N. gonorrhoeae* LtgA differentially activate NOD1 and NOD2. Multimeric and monomeric PGN was prepared using recombinant LtgA or human neutrophil lysozyme (as described in Experimental Procedures) and quantity in the preparations was normalized to diaminopimelic acid content. (A) A representative standard curve of o-phthalaldehyde fluorescence plotted against amino acid concentration using phenylalanine (red triangles) or isoleucine (blue circles) standards is shown. The fluorescence and interpolated concentration of four monomeric PGN preparations upon their initial reconstitution in water is noted by open diamond symbols.

Receptor activation was compared between the indicated PGN preparations using reporter cell lines described in Figure 2 using overnight stimulation of the cells with a concentration of PGN correlating with 8.5 μ M of diaminopimelic acid equivalents: (B) TLR2, (C) NOD1, (D) NOD2, and (E) Null1 HEK293-Blue™ cells. (F) NOD1- and (G) NOD2-expressing reporter cells were treated with the indicated LtgA- or lysozyme-liberated PGN monomers at the indicated concentrations. Data shown are representative of at least two experiments from at least two independent preparations of PGN monomers. Data shown represent mean values $\pm$ S.E.M. from cells treated with two preparations of PGN 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 4.

The predicted structures of the major products of digestion of *N. gonorrhoeae* peptidoglycan by LtgA or lysozyme. The structure of 1,6-anhydro-MurNAc-containing PGN monomers is predominantly released by LtgA (left) and reducing-MurNAc-containing PGN monomers is predominantly released by lysozyme (right).

Figure 5.

LtgA-generated 1,6-anhydro-MurNAc-containing PGN monomers are poor NOD2 activating ligands. The indicated PGN monomer species were prepared as described using recombinant LtgA or human neutrophil lysozyme as described in the methods and isolated using HPLC. The PGN monomeric species collected are indicated on chromatograms from the HPLC runs (A). PGN monomer structures were verified by mass spectroscopy (B). The monomer species are defined in (C). The capacity of each to stimulate NOD1 (D) and NOD2 (E) reporter cells was assessed at the indicated concentrations. Data shown are representative of at least two experiments from at least two independent preparations of PGN monomers. (D and E) Data shown represent mean values $\pm$ S.E.M. from cells treated with two preparations of PGN monomers 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.

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