Differential Peptidoglycan Recognition Assay Using Varied Surface Presentations

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KEYWORDS: NLRP1, NOD2, innate immunity, muramyl dipeptide, surface plasmon resonance, peptidoglycan.

ABSTRACT: Bacterial peptidoglycan (PG) is recognized by the human innate immune system to generate an appropriate response. To gain an appreciation of how this essential polymer is sensed, a surface plasmon resonance (SPR) assay using varied PG surface presentation was developed. PG derivatives were synthesized and immobilized on the surface at different positions on the molecule to assess effects of ligand orientation on the binding affinities of NOD-like receptors (NLRs). NLRP1 and NOD2 are cytosolic innate immune proteins known to generate an immune response to PG. Both possess conserved leucine rich repeat domains (LRR) as proposed site of molecular recognition, though limited biochemical evidence exists regarding the mechanisms of PG recognition. Here direct biochemical evidence for the association of PG fragments to NOD2 and NLRP1 with nanomolar affinity is shown. The orientations in which the fragments were presented on the SPR surface influenced the strength of PG recognition by both NLRs. This assay displays fundamental differences in binding preferences for PG by innate immune receptors and reveals unique recognition mechanisms between the LRRs. Each receptor uses specific ligand structural features to achieve optimal binding, which will be critical information to manipulate these responses and combat diseases.

The innate immune system is the first line of defense against microbial pathogens[1]. It is expansive and complex, relying on specific recognition events and concerted signaling pathways to mount the proper response. A major driver of this response is bacterial cell wall component peptidoglycan (PG). While PG is structurally conserved, nature has a variety of strategies to modify the polymer (Figure 1), generating a diverse set of fragments to serve as immunogenic ligands for several classes of receptors, including the cytosolic NOD-like receptors (NLR), transmembrane toll-like receptors (TLR) and peptidoglycan recognition proteins (PGRPs)[2-4]. The diversity of PG ligands and their protein partners creates the need for specificity in recognition and downstream signaling[5-7]. Molecular recognition in NLRs is believed to rely on the leucine-rich repeat domain (LRR), a conserved motif,[8-9] most often associated with protein-ligand and protein-protein interactions[10-13]. All known NLRs with the exception of NLRP10 contain an LRR domain[14]. The mechanisms by which NLRs bind PG ligands are not understood, though are crucial in understanding misrecognition events that lead to the development of autoimmune diseases. Two NLRs known to induce immune activation in response to PG are NOD2 and NLRP1.

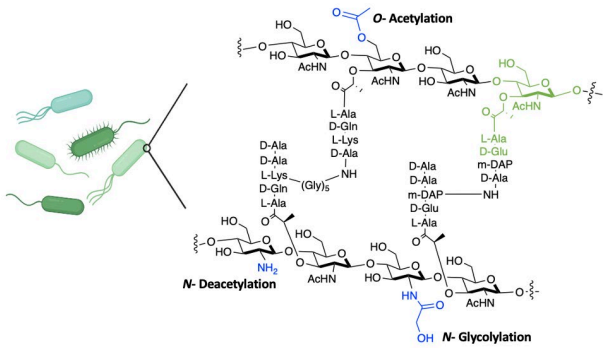

Figure 1. Structure of PG. The carbohydrate core is comprised of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), with a pentapeptide stem off the C3 position of MurNAc that crosslinks to the parallel strand[15-16]. Many bacteria modify PG, shown in blue, generating a diverse pool of fragments[17-18]. Minimal immunostimulatory fragment muramyl dipeptide (MDP) is highlighted in green.

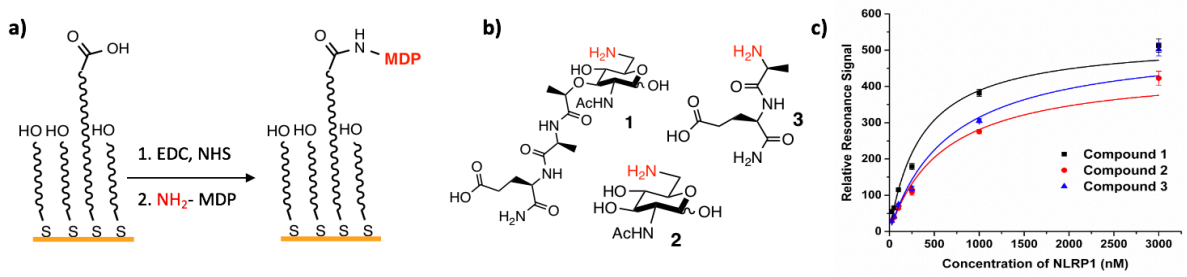

Figure 2. The LRR domain of NLRP1 binds MDP and its carbohydrate and peptide components. a) Schematic of SPR surface which acts as a scaffold for differential PG fragment attachment. Carboxylic acid terminated SAMs are activated with EDC/NHS and coupled to amine functionalized derivatives b) Structures of 6-amino MDP (**1**), 6- amino GlcNAc (**2**), L-ala-D-isoglutamine dipeptide (**3**) c) Binding curves for NLRP1 LRR to compounds **1-3**

Table 1. Surface K_D values for NLRP1/ NOD2 LRRs to different orientations of MDP

		Surface K_D (nM)	
Number	**Compound**	**NOD2**	**NLRP1**
1	6-amino MDP	213±24*[30]	362±40
2	6 -amino GlcNAc	354±40*[30]	606±78
3	L-ala D-iso dipeptide	428±49*[30]	668±147
4	2-amino MDP	1700±5	110±10
5	Peptide amino MDP	920±90	350±20
6	1-amino MDP	700±100*[37]	560±70

*Denotes previously reported surface K_D values for the NOD2 LRR

Mutations in both cause increased susceptibility to a variety of diseases, including Crohn's disease and Vitiligo[19-21]. Both proteins activate an immune response to synthetic PG fragment MDP[22-24]. NOD2 utilizes the NF-κb and MAP kinase signaling pathways[25-26], while NLRP1, the first discovered inflammasome forming NLR,[27] activates caspase-[22, 28-29]. Although it has previously been shown that MDP is a ligand for NOD2[23, 26, 30-33], limited biochemical evidence exists showing that MDP and NLRP1 interact[22]. Here, the NLRP1 LRR domain was expressed as a tag-free LRR (Figure S1). This construct was shown to be α-helical in character by circular dichroism spectroscopy (Figure S2), agreeing with its crystal structure (PDB 4IM6). Due to the ubiquitous nature of LRR domains in molecular recognition and lack of evidence for a PG-NLRP1 interaction, the NLRP1 LRR binding to MDP was tested using SPR (Figure 2a). In this assay, amine functionalized PG derivatives are coupled to carboxylic acid terminated self-assembled monolayers (SAM) and the change in refractive index is measured as a function of increasing protein concentration (Figure 2c)[26, 34]. However, we sought a way to assess binding of NLRP1 to MDP and simultaneously gain information on the binding site structure. As no ligand-bound structure exists[35], we manipulated the ligand's structure rather than the receptor. To this end, an expanded SPR assay was developed in which ligand presentation on the SAM surface could be modified to asses binding to PG fragments in different orientations.

First, a collection of amine functionalized MDP derivatives was synthesized, with functionality on the C6 (**1**), C2 (**4**), D-isoglutamine (**5**), and C1 (**6**) positions (Figure 2b, Figure 3a). For gaining binding site structural information, this method provides critical advantages, as it allows the selective exposure of certain faces of the ligand to the protein of interest, blocking others via surface attachment. We envisioned that assessment of binding between the NLR and different ligand orientations would provide a map of critical contact regions. Access to the C6 (**1**), C1 (**6**) and C2 (**4**) derivatives was achieved using previously developed syntheses[36-37] . The new peptide linked derivative (**5**) was synthesized from *N*-acetylglucosamine (SI). MDP tethered at the C6 (**1**), and its carbohydrate (**2**) and peptide (**3**) components were chosen as the first presentations to assess NLRP1 binding, as these orientations had been previously shown to bind the NOD2 LRR[26, 30]. To assess non-specific binding, all PG functionalized gold chips contained an ethanolamine control lane. BSA was also run over the MDP functionalized surface and no binding was observed (Figure S6).

It was determined that the NLRP1 LRR binds (**1**) with a surface K_D of 362± 40 nM; providing the first direct biochemical evidence that MDP is a ligand for NLRP1. This confirms that like NOD2, NLRP1 can recognize Gram (+) and Gram (-) PG, as MDP is a conserved structure in both bacteria[24, 38]. The LRR was able to bind individual carbohydrate (**2**, 606±78 nM) and peptide (**3**, 668±147 nM) components of MDP, with affinity decreasing 2-fold for both fragments compared to intact MDP. These data indicate that while the carbohydrate and peptide are not individually required for NLRP1 binding, the presence of both enhances affinity, as observed for NOD2[30].

There is little data indicating which residues on NLRP1 are critical for PG binding, making mutagenesis or docking studies on the LRR difficult. By immobilizing MDP at varying tether points (**4, 5, 6**), different faces of the molecule were exposed for recognition, providing an image of crucial protein-ligand interaction sites that has previously been unavailable. SPR analysis revealed the LRR binds MDP tethered at the C2 position (**4**) with the highest affinity (110±10 nM), indicating attachment at this position presents MDP in the optimal orientation for molecular recognition (Figure 3b). The peptide linked (**5**) and C6 linked MDP (**1**) bind with approximately the same affinity, 350±20 nM and 362 ± 40 nM respectively (Figure 2c-3c). These data suggest that the C2 and C6 positions are most likely solvent exposed and may not form crucial binding interactions within the binding pocket. Interestingly, MDP presented at the C1 position (**6**) bound to NLRP1 with the lowest affinity of all MDP derivatives tested (560±70nM) (Figure 3d). This is complementary to binding between the NOD2 LRR and C1 linked MDP, as affinity dropped significantly between the C6 and C1 tethered MDPs (Table 1)[37]. These data demonstrate the important role of the free anomeric in binding of PG to both NLRs. The well-studied importance of polar amino acids within carbohydrate binding sites suggests this hydroxyl participates in critical hydrogen bonding interactions that stabilize and increase the affinity of NLRs for the sugar moiety[39-40]. Further structural characterization will be required to determine the precise role of the anomeric hydroxyl in recognition.

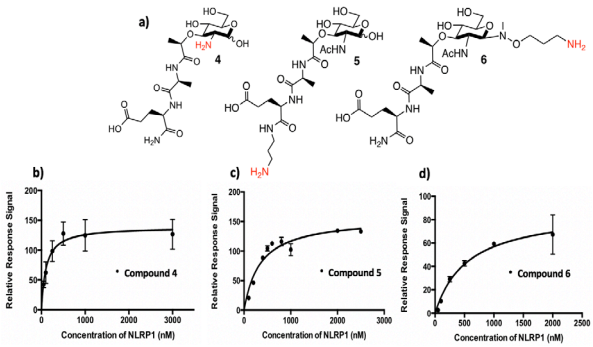

Figure 3. LRR domain of NLRP1 binds MDP when presented in different orientations. a) Structures of amine derivatives b) binding curve for compound (**4**) c) (**5**) d) (**6**).

To further test the ability of this assay to decipher subtle differences in ligand requirements of NLR binding, binding of NOD2 to the remaining MDP orientations (**4** and **5**) was tested. Testing NOD2 binding gave the chance to compare binding requirements between two proteins recognizing the same ligand. Interestingly binding affinities for the NOD2 LRR to the PG tethered library are quite different than those observed for NLRP1 (Table 1). For MDP tethered by the peptide (**5**), NOD2 LRR bound MDP with lower affinity (920±90 nM) than when presented at the C6 (**1**) and C1 (**6**) positions (Figure S5). Interestingly, NOD2 bound the C2 tethered MDP (**4**) with the lowest affinity of all orientations tested, with a surface K_D of 1700±5 nM (Figure 3b). This is the opposite of NLRP1, whose LRR bound this orientation the tightest, with a surface K_D over 15 times lower than that for NOD2 (Table 1). In order to demonstrate binding is specific to PG derived fragments, galactosamine and L-alanine tripeptide were tethered to the SPR surface. Both NOD2 and NLRP1 did not exhibit binding to these non-PG derived carbohydrate and peptide compounds (Figure S8).

NOD2's decreased affinity for (**4**) was expected[36], as prior mutagenic analysis predicts the C2 acetate forms a hydrogen bond with a R877 residue in the LRR pocket. Mutation of R877 to alanine resulted in a 4-fold decrease in affinity of NOD2 for MDP, confirming this key binding interaction[30]. Surface attachment at this position could sterically block hydrogen bond formation. To accommodate MDP tethered at the C2, NOD2 would hypothetically bind from the opposite face of the molecule, an energetic penalty, significantly decreasing the affinity. NLRP1's significantly higher affinity for (**4**) suggests that the C2 position is solvent exposed within the binding site, and that NLRP1's key interactions with PG ligands are unique from NOD2.

Unlike NLRP1, NOD2 recognition appears to rely on the free terminal amide in the D-isoglutamine of MDP. NOD2's low affinity for (**5**) is in agreement with our previous computational studies, in which the peptide chain sits within a binding cleft (Figure S7).

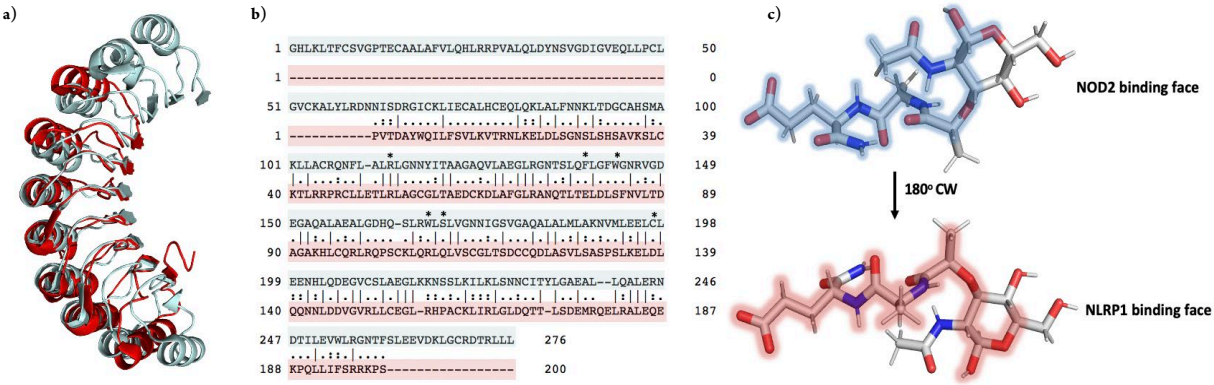

Figure 4. Optimal PG binding of NOD2 and NLRP1 to MDP requires different ligand orientations. a) Crystal structure alignment of human NLRP1 LRR (PDB 4IM6, aa 791-990) and Rabbit NOD2 LRR (PDB 5IRN, aa 765-1040). b) EMBOSS Needle pairwise sequence alignment of the LRRs' reveals a 22.5% sequence identity. Asterisks denote putative NOD2 binding site residues c) Structural model highlighting critical contact regions of NOD2 (blue) and NLRP1 (red) based on collective SPR analysis. Both NLRs appear to recognize different faces of the molecule to achieve optimal binding.

Presenting MDP through this position would prevent the peptide from forming favorable interactions along the cleft, forcing MDP into an unfavorable conformation for NOD2 recognition. Previous studies have shown that extension of this peptide chain abolishes NOD2's NF-kB activation[41]. NLRP1's higher affinity for (**5**) suggests the peptide is solvent exposed within the binding site. This is supported by the fact that NLRP1 has lower affinity for the peptide (**3**) than NOD2, (668±147nM vs 428±49 nM, respectively) suggesting NOD2 makes more critical contacts with the peptide stem, and is able to maintain tighter binding in the absence of the sugar[30]. While the LRR domains share highly α-helical secondary structures (Figure 4a), sequence alignment reveals only a 22.5% sequence identity (figure 4b). Importantly, this proposed aromatic rich binding site on NOD2 has no complement in NLRP1. Lack of a common binding motif and binding specificities indicate that while both NLRs recognize MDP, they use different mechanisms. Based on the collective SPR analysis of PG fragments in a suite of different orientations, a structural model of the two NLRs binding preferences was developed (Figure 4c). It appears that NOD2 requires interaction with the anomeric and C2 positions of the sugar, recognizing PG best when presented via the C6, which exposes the full peptide chain and C2 *N*-acetyl position (Figure 4c). In contrast, while NLRP1 appears to require the free anomeric hydroxyl, optimal binding occurs on the opposite face of the sugar ring(Figure 4c).

Use of SPR as a method to study carbohydrate-protein interactions has been used extensively by our lab and others[26, 30-31, 42]. Development of an assay to vary attachment of PG to the SPR surface taught us a variety of important features regarding NLR recognition. Prior to these studies, limited evidence that NLRP1 directly interacted with MDP was available. The data presented here clearly demonstrate that NLRP1 binds to MDP with nanomolar affinity via its LRR domain (Table 1). We have shown that differential surface presentation can also be used to gain information on subtle difference in ligand structural requirements for innate immune receptors. Such information would be unattainable using other binding assays, particularly those in solution where ligand orientation cannot be controlled. Synthesis of amine functionalized PG fragments allowed for site specific ligand attachment to a sensitive gold surface, exposing select faces of the molecule for recognition, revealing that there are critical regions on PG recognized for optimal binding, and more importantly suggests that the binding pockets used in molecular recognition differ between NLRs. Subtle differences in ligand orientation have a major effect on NLR binding, and knowledge of these requirements will prove instrumental in rational design of immunogenic molecules. The use chemically modified ligands to determine SAR have been widely used in medicinal chemistry, particularly in solution-based assays such as methyl scanning[43-44]. While SPR and other surface-based binding assays are also commonly used in drug design, they often involve protein or lipid surface attachment[45-47]. Our ligand-based approach allows for a more molecular level assessment of optimal drug-protein interaction sites, aiding in rational ligand design and optimization. The "tethering" SPR-technique has the potential to be extend beyond PG-NLR interactions, to presentation of drug molecules whose targets have therapeutic implications, such as oncogenic kinases KRAS[48], or viral capsid proteins. This deeper understanding of discrete structural requirements for the proteins and ligands involved in recognition will be critical in understanding how the immune system responds to PG, and how misrecognition events can lead to development of autoimmune disorders.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures and materials including cloning details, SPR details and sensograms, synthetic procedures and protein characterization

The Supporting Information is available free of charge on the ACS Publications website.

brief description (file type, i.e., PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest

ACKNOWLEDGMENT

We are thankful for support from the Delaware COBRE program, supported by a grant from the National Institute of General Medical Sciences (NIGMS 1 P30 GM110758 and 1 P20 GM104316-01A1) from the National Institutes of Health. C.L.G. is a Pew Scholar in the Biomedical Sciences, supported by the Pew Charitable Trusts. We gratefully acknowledge the National Science Foundation (CAREER CHE 1554967) for support of this research. M.L.L. thanks the NIH for support through a CBI training grant: 5T32GM008550. We thank our colleagues at the University of Delaware: Dr. Papa Nii Asare-Okai, the Director of the MS facility, and Dr. Shi Bai at the NMR facility at the University of Delaware. We thank professor Jenny Ting at UNC Medical School for the initial NLRP1 plasmid

ABBREVIATIONS

NLRP1, NACHT, LRR and PYD domains-containing protein 1; NOD2, nucleotide-binding oligomerization domain-containing 2; MDP, muramyl dipeptide; LRR, leucine-rich repeat; SPR, surface plasmon resonance; SAM, self-assembled monolayer

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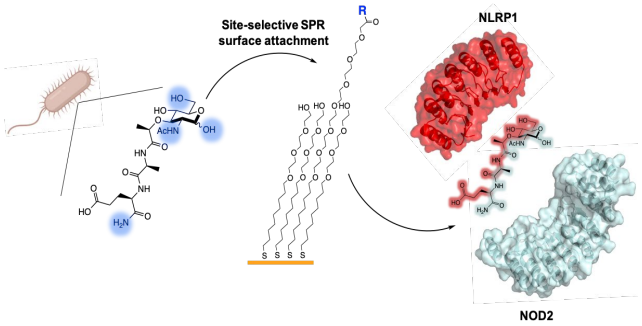
Site-selective SPR
surface attachment
R
NLRP1
NOD2