

## Peptidoglycan Signaling in Innate Immunity and Inflammatory Disease\*

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Christine McDonald<sup>‡</sup>, Naohiro Inohara,  
and Gabriel Nuñez<sup>§</sup>

From the Department of Pathology and Comprehensive  
Cancer Center, the University of Michigan Medical  
School, Ann Arbor, Michigan 48109

The ability to detect harmful pathogens and eradicate them is essential for the survival of an organism. One of the first lines of defense is the innate immune system, a broad specificity inflammatory response characterized by the recruitment and activation of phagocytes. This type of immune response is well conserved from invertebrates through higher mammals and is stimulated by the recognition of conserved components of microorganisms called pathogen-associated molecular patterns (PAMPs).<sup>1</sup> These PAMPs consist of molecules not found in the host, including bacterial cell wall components such as peptidoglycan (PGN). Recognition of PAMPs by specific proteins called pattern recognition molecules (PRMs) activates inflammatory signaling pathways and the stimulation of an immune response. Recent studies challenging the role of Toll-like receptor 2 (TLR2) as the major PGN receptor, the genetic dissection of the role of mammalian peptidoglycan response proteins (PGRPs) in immune responses, and the identification of Nod1, Nod2, and cryopyrin as PGN response proteins have put the spotlight on the mechanisms of PGN recognition and signaling in innate immunity. In addition, mutations found in Nod2 and cryopyrin are genetically associated with the development of specific inflammatory diseases. This review will focus on recent findings about the proteins involved in binding to and stimulating an intracellular signaling response to PGN and their relationship to inflammatory disease.

### Peptidoglycan Is an Immunostimulatory Component of the Bacterial Cell Wall

PGN is an essential and unique component of bacteria that provides rigidity and structure to the bacterial cell wall (reviewed in Ref. 1). Virtually all bacteria contain a layer of PGN, but the amount, location, and specific composition vary. For example, PGN is found as a thick exposed layer comprising the bacterial cell wall of Gram-positive bacteria in association with lipoteichoic acid, whereas in Gram-negative bacteria it is present as thin layer overlaid by a thick layer of lipopolysaccharide (LPS). PGN is composed of carbohydrate chains of  $\beta(1-4)$ -linked, alternating

*N*-acetylglucosamine and *N*-acetylmuramic acid sugars cross-linked by short peptide chains with alternating L- and D-amino acids (Fig. 1). These peptide chains (called stem peptides) contain unique amino acids such as D-glutamic acid connected by its  $\gamma$ -carboxyl group instead of the usual  $\alpha$  group (D- $\gamma$ -Glu) and *meso*-diaminopimelic acid (mDAP). Two major types of PGN can be classified by the nature of the third residue of the stem peptide (Fig. 1). In Gram-negative bacteria, this residue is primarily mDAP (DAP-PGN), and in Gram-positive bacteria, the third residue is variable but is commonly lysine (Lys-PGN). However, there is considerable variation in the modes of cross-linking between stem peptides as well as the residues included in the interpeptide bridges, so the results from studies with Lys-PGN or DAP-PGN may not correlate to all Gram-positive or Gram-negative bacteria, respectively.

It has been known for a long time that PGN promotes an inflammatory response (reviewed in Ref. 2). Initially this was shown in animal models where intraperitoneal injection of PGN from Gram-positive bacteria into rats promoted inflammation and subsequent arthritic destruction of their joints. Later, a subcomponent of PGN, muramyl dipeptide (MDP), was found to be the minimal chemical structure required for the adjuvant activity of Freund's complete adjuvant. In cell culture models, PGN has been demonstrated to stimulate the production of inflammatory cytokines, such as IL-6, IL-1 $\alpha/\beta$ , and TNF $\alpha$  in monocytes, macrophages, neutrophils, and epithelial cells.

### Peptidoglycan Signaling in Host-Microbial Interactions

There are several classes of PRMs that include scavenger receptors, TLRs, and nucleotide-binding oligomerization domain-containing proteins called Nods. Scavenger receptors are found in the extracellular space and primarily function to opsonize microbes to enhance their clearance through phagocytosis. The TLRs are a family of transmembrane receptors that recognize specific PAMPs and can generate an immune response in response to this recognition (reviewed in Ref. 3). The most recently identified PRM class is the Nod family, which are intracellular sensors of PAMPs (reviewed in Ref. 4).

PRMs that detect and respond to PGN include PGRPs and specific members of the Nod family (Nod1, Nod2, and cryopyrin). Initially, TLR2 was characterized as the receptor for PGN through analysis of TLR2-deficient mice (5). Macrophages from TLR2-deficient mice lost the ability to secrete the inflammatory cytokines TNF $\alpha$  and IL-6 in response to PGN from Gram-positive bacteria. These studies, combined with results from TLR2 overexpression studies, led investigators to conclude that TLR2 is essential for responsiveness to PGN. However, one recurring problem in this field is that many bacterial cell wall components are covalently linked to one another, resulting in the co-purification of other immunostimulatory molecules in many commercial sources of "pure" PAMPs. In light of this, a recent paper prepared highly purified PGN from several strains of bacteria, and at different steps in the purification procedure the purified PGN was tested for recognition by TLR2. The results from this study demonstrated that TLR2 is not a sensor for PGN but is activated by the lipoproteins and lipoteichoic acids that are commonly present in these commercial PGN preparations (6).

The recognition of PAMPs by PRMs leads to the activation of intracellular signaling pathways (Fig. 2). One pathway activated by PRMs is the NF- $\kappa$ B pathway (reviewed in Ref. 7). Through different upstream adaptor molecules and kinases, PRMs activate a complex of protein kinases called I $\kappa$ B kinases (IKKs). Subsequently, the IKK complex activates the transcription factor NF- $\kappa$ B, which initiates the transcription of pro-inflammatory genes. The mitogen-activated protein kinase (MAPK) signaling pathway also contributes to the expression of pro-inflammatory genes through the activation of JNK, Erk-1, and p38 kinases and their down-

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<sup>§</sup> To whom correspondence should be addressed. Tel.: 734-764-8514; Fax: 734-647-9654; E-mail: bclx@umich.edu.

<sup>1</sup> The abbreviations used are: PAMP, pathogen-associated molecular pattern; PGN, peptidoglycan; PRM, pattern recognition molecule; PGRP, peptidoglycan response protein; LPS, lipopolysaccharide; mDAP, *meso*-diaminopimelic acid; iE-DAP, D- $\gamma$ -glutamyl-*meso*-DAP; MDP, muramyl dipeptide; IL, interleukin; TNF, tumor necrosis factor; TLR, Toll-like receptor; IKK, I $\kappa$ B kinase; MAPK, mitogen-activated protein kinase; JNK, N-terminal c-Jun kinase; CARD, caspase recruitment; NOD, nucleotide-binding oligomerization domain; LRR, leucine-rich repeat; CD, Crohn's disease; BS, Blau syndrome; EOS, early onset sarcoidosis; MWS, Muckle Wells syndrome; FCAS, familial cold autoinflammatory syndrome; CINCA, chronic infantile neurological cutaneous and articular syndrome; RICK, RIP-like interacting CLARP kinase.

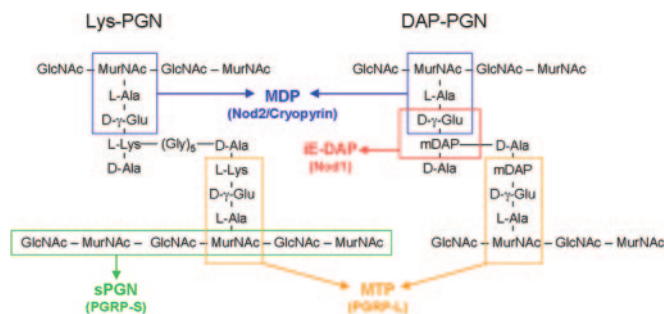


FIG. 1. **Schematic of PGN structure.** Shown are two examples of the general types of PGN, Lys-PGN (from the Gram-positive bacterium, *S. aureus*, strain Copenhagen) and DAP-PGN (from Gram-negative bacteria such as *Escherichia coli*). Ligands for Nod1 (red box), Nod2, and cryopyrin (blue boxes) are shown. The putative binding sites for PGRP-S (green box) and PGRP-L (orange boxes) are indicated. MTP, muramyl tripeptide; sPGN, soluble PGN; GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid.

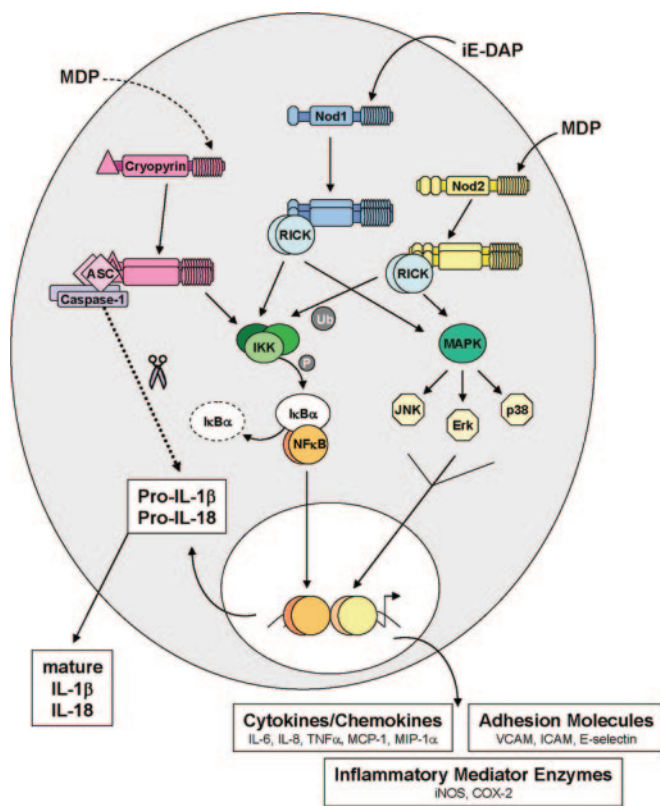


FIG. 2. **Signaling pathways activated by peptidoglycan-derived ligands.** Stimulation of cells with iE-DAP causes the oligomerization of Nod1 and recruitment of the protein kinase RICK. RICK activates the IKK complex, which then phosphorylates (*P*) IκBα, signaling its degradation by the proteasome. Once released from its inhibitor IκBα, the transcription factor NF-κB translocates to the nucleus and initiates the transcription of pro-inflammatory genes. Nod1 and RICK also activate the MAPK-stimulated pathways JNK, Erk, and p38 that result in the activation of specific transcription factors, such as AP-1, SRF, and CREB. Nod2 is activated by MDP stimulation of cells and, like Nod1, oligomerizes and recruits RICK, which activates the IKK complex and stimulates NF-κB activation by ubiquitinylation (*Ub*) of the IKK $\gamma$  subunit of the IKK complex. Nod2 also activates the MAPK pathway upon stimulation. Activation of cryopyrin by its proposed ligand, MDP, causes the formation of a complex composed of cryopyrin, ASC, CARD8, and caspase-1, which activates the IKK complex and NF-κB signaling, as well as induces maturation and secretion of pro-IL-1 $\beta$  and pro-IL-18 through proteolytic cleavage by caspase-1.

stream targets (reviewed in Ref. 8). Another intracellular signaling pathway commonly stimulated by PRMs activates a pro-inflammatory caspase, caspase-1, that acts to process pro-IL-1 $\beta$  and pro-IL-18 to active forms for secretion.

### Peptidoglycan-binding Proteins

PGRP was first identified in the silk worm, *Bombyx mori*, through purification of a protein from hemolymph that binds to Gram-positive bacteria and activates an immune response (9). In

insects, PGRPs can be classified into two classes, short PGRPs (PGRP-S) and long PGRPs (PGRP-L) (reviewed in Refs. 10 and 11). The PGRP-S class is thought to be composed of secreted proteins, whereas the PGRP-L class is predicted to be a family of intracellular or transmembrane proteins. Many PGRPs have been shown to bind directly to PGN, each with distinct preferences for binding Lys-PGN or DAP-PGN. This binding discrimination to Lys-PGN or DAP-PGN leads to activation of specific immune signaling pathways (Toll and Imd, respectively, in *Drosophila*). This mechanism of using Lys-PGN and DAP-PGN to activate distinct intracellular signaling pathways is shared with mammalian Nod proteins (see below). All PGRPs contain a conserved region, called the PGRP domain, that has homology to bacteriophage T7 lysozyme and bacterial type 2 *N*-acetylmuramoyl-L-alanine amidase and are predicted to have amidase activity. PGRPs play essential roles in insect innate immunity and either act upstream of the insect immune signaling pathways Toll and Imd to activate antimicrobial responses or act as scavenger receptors for PGN to down-regulate the immune response.

The characterization of insect PGRPs as molecules involved in innate immunity led to the identification and cloning of mammalian PGRP orthologues (12, 13). The mammalian PGRP family is composed of four members, PGRP-S, PGRP-I $\alpha$ , PGRP-I $\beta$ , and PGRP-L (reviewed in Refs. 11 and 14). All mammalian PGRPs have been shown to be secreted proteins and to bind directly to PGN as well as live bacteria. However, even though all four members have the PGRP domain, only one mammalian PGRP, PGRP-L, has been reported to have amidase activity. PGRP-L binds to the muramyl tripeptide moiety in PGN and cleaves between the muramic acid and the first amino acid of the stem peptide chain, L-alanine (Fig. 1). This specificity is identical to the PGN degradation enzyme, serum *N*-acetylmuramoyl-L-alanine amidase, leading investigators to conclude that they are the same enzyme (15, 16). Mammalian PGRPs also have distinct expression patterns, with PGRP-S found in polymorphonuclear leukocytes and bone marrow, PGRP-I $\alpha$  and PGRP-I $\beta$  found in the esophagus, and PGRP-L expressed in the liver, suggesting that they each may play a unique role in host defense.

In contrast to the insect PGRPs, mammalian PGRPs have no known role in stimulating intracellular signaling pathways but rather appear to act as antibacterial or scavenger molecules. Mice deficient in PGRP-S or PGRP-L have been generated, and analysis of these mice has further clarified the physiological roles of these proteins in the immune response. PGRP-S-null mice have normal inflammatory responses (*i.e.* cytokine production), and their neutrophils have normal phagocytic uptake of bacteria. However, these mice are defective in the intracellular killing of nonpathogenic Gram-positive bacteria, possibly because of a lower induction of oxidative burst (17). The results from these mice demonstrate that PGRP-S is an intracellular PGN-binding protein that has a non-enzymatic antibacterial effect in neutrophils but does not act as an effector molecule to generate an immune response in response to PGN. In contrast to PGRP-S, the phenotype of PGRP-L-null mice is surprisingly mild, and responses to PGN and Gram-positive organisms were unimpaired, suggesting that PGRP-L does not play an essential role in mammalian innate immunity (18).

### Nod Family of Proteins

The Nod family of proteins was originally identified in a GenBank™ data base search for molecules with homology to the apoptosis regulator Apaf-1 (reviewed in Ref. 4). These cytoplasmic proteins have a similar domain structure with amino-terminal effector binding domains, such as caspase recruitment (CARD), pyrin, or baculovirus inhibitor of apoptosis domains, a central nucleotide-binding oligomerization domain (NOD), and a carboxyl-terminal ligand sensing domain consisting of leucine-rich repeats (LRRs) or WD40 repeats. It is unknown at this time if Nods bind directly to their ligands, similar to the PGRPs, or if ligand sensing requires additional molecules or co-receptors. It is known, however, that specific residues within the LRRs of Nod1 and Nod2 are required for ligand sensing (19). Intracellular signaling of Nods is generated by the clustering of downstream signaling molecules interacting with the effector binding domains through the oligomerization of Nod proteins via interactions of the central NOD.

The activating ligands for only 3 of the 23 Nod family members have been identified so far, and all of these ligands are components of PGN (see below).

### Nod1

Nod1 protein is widely expressed in mammals and has been shown to be an essential intracellular sensor of specific types of bacteria through the detection of specific moieties found in its PGN structure (reviewed in Ref. 4). LPS preparations were demonstrated to activate Nod1, but later studies determined that Nod1-dependent activation of NF- $\kappa$ B was because of PGN co-purification with the LPS (20). The real ligand of Nod1 was characterized as PGN fragments that contain the dipeptide, D- $\gamma$ -glutamyl-*meso*-DAP (iE-DAP), which can either be generated by degradation of PGN or secreted by bacteria during replication (21, 22). The mDAP residue is found only in Gram-negative and specific Gram-positive bacteria, indicating that Nod1 is involved in recognition of a specific subset of bacteria and is not a general sensor of PGN (Fig. 1).

Nod1 activates NF- $\kappa$ B through recruitment and oligomerization of RICK (also called RIP2 or CARDIAK), which results in the activation of the IKK complex (23). Recently, it has been demonstrated that Nod1 is able to sense and respond to an extracellular Gram-negative pathogen, *Helicobacter pylori*, through the injection of PGN by *H. pylori* into the cytosol of epithelial cells in a type IV secretion apparatus-dependent mechanism (24). These results may provide a novel mechanism for detecting a noninvasive pathogen and may explain how epithelial cells can discriminate between pathogenic and commensal bacteria.

### Nod2

Nod2 is an intracellular sensor of PGN that plays an essential role in intestinal mucosal immunity (reviewed in Ref. 4). Mutations in the *NOD2* gene have been correlated with three inflammatory diseases, Crohn's disease (CD), Blau syndrome (BS), and early onset sarcoidosis (EOS) (25–28). Nod2 has a restricted expression pattern, primarily expressed in monocytes and specialized epithelial cells found in the crypts of Lieberkühn called Paneth cells (29–31). Additional reports now demonstrate a broader range of expression, with Nod2 expressed in granulocytes, neutrophils, and dendritic cells, as well as stimulated intestinal epithelial cells and myofibroblasts (32–34). It is unknown at this time if Nod2 functions similarly in all these cell types or if Nod2 plays different specific roles in each of these cells.

Initially, Nod2 was thought to be activated by several types of LPS and PGN from *Staphylococcus aureus*. However, it was later found that the LPS-induced activation of Nod2 was a result of co-purifying PGN in the LPS preparations. The specific moiety of PGN that stimulates Nod2 activity is MDP (Fig. 1). MDP is a common component of almost all types of PGN, indicating that Nod2 acts as a general sensor of PGN (22, 35, 36). Once activated by MDP, Nod2 activates downstream inflammatory signaling pathways through RICK (29). In the Nod2 signaling pathway, RICK was shown to activate NF- $\kappa$ B by promoting the ubiquitinylation of the IKK $\gamma$  subunit of the IKK complex (37).

Two groups have generated Nod2-deficient mice and found that macrophages from these mice do not activate inflammatory signaling pathways or cytokine production in response to stimulation with MDP (38–40). Nod2-deficient mice have an increased susceptibility to oral infection with *Listeria monocytogenes*, which correlates with a loss of expression of some intestinal antibacterial peptides. These results may explain the intestinal pathology seen in CD, an inflammatory bowel disease associated with loss-of-function Nod2 mutations, as CD patients also have decreased expression of these antibacterial peptides (41). Surprisingly, these mice have normal intestinal pathology, so the Nod2 mutations found in CD may act as a promoting factor for disease development and not as an initiating factor. A controversial result from these mice suggests that Nod2 acts as a negative regulator of TLR2 signaling in splenocytes (39). However, two other studies do not see the same phenotype in their experiments with bone marrow-derived macrophages and in fact see a synergistic response of MDP and TLR2 stimulation (38, 40), suggesting that the inhibitory function of Nod2 may be cell type-specific and not a universal characteristic of Nod2.

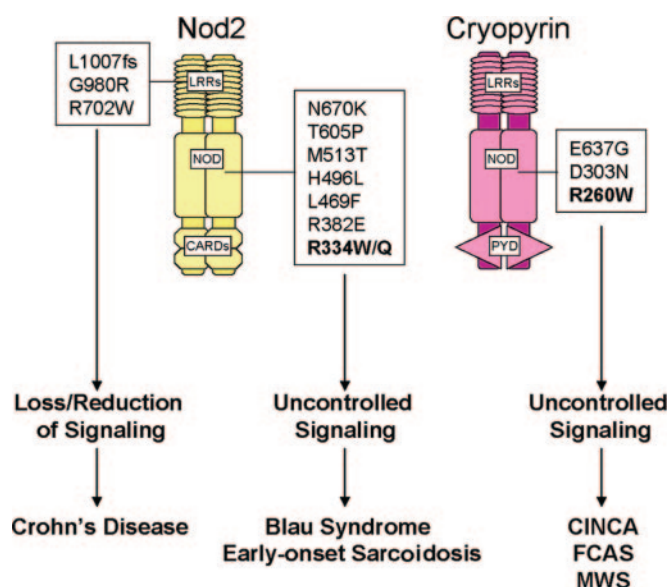


Fig. 3. Inflammatory disease-associated mutations in Nod2 and cryopyrin. Crohn's disease associated mutations found mainly in the LRRs of Nod2 result in decreased or a loss of activity. Mutations clustered in the NOD of Nod2 and cryopyrin cause constitutive activation of these proteins. Uncontrolled signaling of Nod2 is associated with Blau syndrome and early onset sarcoidosis. The hereditary fever syndromes, MWS, FCAS, and CINCA, are caused by specific mutations in the cryopyrin NOD. The mutations shown in *bold* are similar missense mutations in analogous positions in the two proteins.

### Cryopyrin

The most recently reported sensor of PGN is the Nod family member, cryopyrin (also called Nalp3 or PYPAF1). Cryopyrin is the protein product of the gene *CIAS1*, which is mutated in several hereditary fever syndromes and is expressed primarily in polymorphonuclear cells and chondrocytes (reviewed in Ref. 42). Cryopyrin has been shown to be a component of a protein complex consisting of cryopyrin, the adaptor molecules ASC and cardinal/CARD8, and caspase-1 (43). The oligomerization of these proteins causes the processing of IL-1 $\beta$  by caspase-1, as well as the activation of NF- $\kappa$ B (43, 44). Recently, a study performed in an ectopic expression system suggested that the activator of cryopyrin is MDP, the same PGN-derived activator as Nod2 (45). However, studies in a more physiologically relevant system need to be performed to confirm that MDP is the ligand for cryopyrin.

### Peptidoglycan Signaling Proteins and Disease

Genetic mutation of Nod2 and cryopyrin has been associated with the development of several inflammatory diseases. Mutations in Nod2 are associated with CD, BS, and EOS (25–28). The group of hereditary fever syndromes, Muckle Wells syndrome (MWS), familial cold autoinflammatory syndrome (FCAS), and chronic infantile neurological cutaneous and articular syndrome (CINCA), are all due to mutation of cryopyrin (reviewed in Ref. 42). These diseases can be grouped into two classes, autosomal recessive (CD) and dominant (BS, EOS, MWS, FCAS, CINCA) disease, which correlates with loss-of-function and gain-of-function mutations, respectively (Fig. 3). Interestingly, the loss-of-function mutations are found mainly in the LRRs, whereas the gain-of-function mutations cluster in the NOD. In fact, analysis of the autosomal dominant inflammatory diseases has identified a similar missense mutation in analogous positions in either Nod2 (R334W for BS and EOS) or cryopyrin (R260W for FCAS and MWS), suggesting a common molecular mechanism for the development of these inflammatory disorders (46).

### Conclusions

The identification and clarification of the molecules responsible for recognizing and responding to PGN has widened our view on the essential components of a healthy immune response. Essential roles for Nod1, Nod2, and PGRP-S in the immune responses generated by PGN have been shown through analysis of specific gene-deficient mice. Nod2 and cryopyrin are also important inflamma-

tory molecules, as mutation of these proteins cause or contribute to inflammatory disease. Many key questions remain to be addressed which include the cell type-specific roles of PGN recognition molecules, the mechanism of how PAMPs gain access to the intracellular space to stimulate Nods, whether Nods recognize PAMPs directly or require additional molecules to sense these stimuli, as well as how these PGN recognition molecules synergize with other PRMs to mount an efficient immune response. Further investigation into the mechanism of action of these molecules will provide insight into not only immune responses but into the mechanisms of some inflammatory diseases.

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