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Review

Recognition of bacterial peptidoglycan by the innate immune system

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Abstract. The innate immune system recognizes microorganisms through a series of pattern recognition receptors that are highly conserved in evolution. Peptidoglycan (PGN) is a unique and essential component of the cell wall of virtually all bacteria and is not present in eukaryotes, and thus is an excellent target for the innate immune system. Indeed, higher eukaryotes, including mammals, have several PGN recognition molecules, including CD14, Toll-like receptor 2, a family of peptidoglycan recognition proteins, Nod1 and Nod2, and PGN-lytic enzymes (lysozyme and amidases). These molecules induce host responses to microorganisms or have direct antimicrobial effects.

Key words. Innate immunity; pattern recognition receptors; peptidoglycan recognition proteins; Nod; Toll-like receptor-2; CD14; muramyl peptides; bacterial cell wall.

Introduction: innate immunity

Innate immunity is the first line of defense against microorganisms in vertebrates and the only defense against microorganisms in invertebrates and plants. It includes cellular components, which are primarily phagocytic and pro-inflammatory cells (neutrophils and macrophages in vertebrates) and humoral components, such as bacteriolytic enzymes (e.g. lysozyme), complement, mannose-binding protein and soluble CD14 [1–3]. The innate immune system is also required for the initiation of efficient adaptive immune responses [3, 4].

The components of the innate immune system that discriminate between microorganisms and self are clonally encoded and are able to recognize conserved motifs found only in microorganisms but not in higher eukaryotes. When present on cells, they are referred to as pattern recognition receptors. In mammals, pattern recognition receptors can induce phagocytosis (e.g. scavenger receptor, or mannan and β-glucan receptors), chemotaxis (e.g. *N*-formyl-methionine receptor) or secretion of pro-inflammatory mediators [e.g. CD14 and Toll-like receptors (TLRs)] [1–3]. Some mammalian pattern recognition receptors (e.g. CD14 or TLR2) have broad specificity and recognize multiple microbial components, whereas others (e.g. TLR9) have narrow specificity [1–3]. Innate immune mechanisms are highly conserved in evolution, and are often similar in vertebrates and invertebrates. For example, both mammals and insects have highly conserved families of TLR receptors, although individual members of these families have different functions in mammals and insects [1–3, 5].

Peptidoglycan: structure and role in bacteria

Peptidoglycan (PGN) is an essential cell wall component of virtually all bacteria [6, 7]. PGN is a polymer of β(1–4)-linked *N*-acetylglucosamine and *N*-acetylmuramic acid, crosslinked by short peptides (fig. 1) [6, 7]. The glycan chain is usually N-acetylated and sometimes O-acetylated, and is relatively similar in all bacteria. The

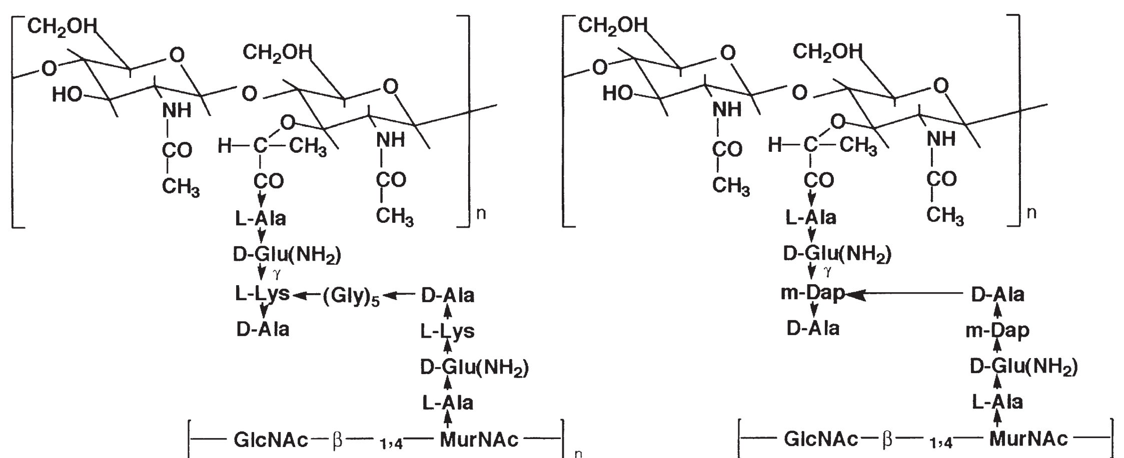

Figure 1. PGN structure. Example of a PGN crosslinked through a pentaglycine interpeptide bridge (*Staphylococcus aureus*) (*left*) and a PGN directly crosslinked through *m*-diaminopimelic acid (*m*-DAP) (from Gram-negative bacteria and Gram-positive rods, e.g. *Escherichia coli* and *Bacillus subtilis*) (*right*).

crosslinking peptides are composed of alternating L and D amino acids and are similar in all Gram-negative bacteria and in Gram-positive bacilli, but usually vary in length and amino-acid composition in Gram-positive cocci [6, 7]. PGN surrounds the cytoplasmic membrane in bacteria and is responsible for maintaining the shape of bacteria and for withstanding the osmotic pressure of the bacterial cell [6, 7]. PGN is especially abundant in Gram-positive bacteria, in which it accounts for approximately half of the cell wall mass and in which other polysaccharides and proteins are covalently bound to it to form a thick cell wall [6–8]. In Gram-negative bacteria, a relatively thin PGN layer surrounds the cytoplasmic membrane underneath the lipopolysaccharide (LPS)-containing outer membrane [6–8]. PGN is a well-known target for almost all clinically useful antibiotics that inhibit bacterial cell wall synthesis [7].

PGN is an excellent target for recognition by the eukaryotic innate immune system because PGN is an essential and unique cell wall component of virtually all bacteria, and because it is not present in eukaryotic cells [6–8]. Indeed, higher eukaryotes have several PGN recognition proteins that induce various host responses to bacteria or have direct antibacterial effects (table 1).

CD14

CD14 is a cell surface glycosylphosphatidylinositol (GPI)-linked 55-kDa glycoprotein highly expressed predominantly on myelomonoctyic cells (including monocytes, macrophages and Langerhans cells) and also at much lower levels on neutrophils and few other non-myelomonocytic cells. Its structure contains 8–10 leucine-rich glycoprotein repeats (LRRs), which are structural motifs that consist of consensus sequences containing two or three repeating leucine residues [9, 10]. CD14 of identical sequence as membrane CD14, but without the GPI anchor, is also present in a soluble form in normal serum and milk.

CD14 functions as the macrophage coreceptor (together with TLR4 and MD-2, see below) for LPS from the outer membrane of Gram-negative bacteria [11]. However, CD14 also functions as the receptor for Gram-positive cell walls and their PGN component [12–15]. The function of CD14 as the PGN receptor is supported by the following evidence [12–19]: (i) activation of CD14-positive cells (monocytes or CD14-transfectants) by PGN is inhibited by anti-CD14 monoclonal antibodies; (ii) PGN-unresponsive CD14-negative cells become responsive after transfection with CD14 and expression of membrane CD14; (iii) CD14 binds to PGN with high affinity (K_D = 25 nM), forms stable complexes with CD14 at approximately 1 : 1 molar ratio, and can completely displace LPS from CD14 and (iv) binding of CD14 to PGN is inhibited by anti-CD14 monoclonal antibodies.

CD14 functions as the cell-activating receptor not only for LPS and PGN, but also for other microbial and synthetic macrophage activators, including lipoteichoic acid (LTA) from Gram-positive bacteria, lipoarabinomannan from mycobacteriae, lipoproteins from spirochetes and mycobacteriae, synthetic lipopeptides, poly β(1–4)-D-mannuronic acid from Gram-negative bacteria, whole cell walls form Gram-positive bacteria, rhamnose-glucose polymer from streptococcal cell walls and synthetic poly β(1–4)-D-glucuronic acid [18, 19]. Therefore, CD14 is a prototypical pattern-recognition receptor that can recognize shared features of a wide variety of microorganisms. However, CD14 also interacts with nonmicrobial ligands, such as phospholipids or mammalian cells undergoing apoptosis [18, 19].

Table 1. Peptidoglycan receptors and recognition molecules*.

Recognition molecule	Main effect	Other ligands (besides PGN)
CD14	activation of macrophages to secrete mediators	LPS, LAM, LTA, bacterial cell walls, lipoproteins, lipopeptides, poly ManU, phospholipids
TLR2	activation of macrophages to secrete mediators	LTA, LAM, lipoproteins, lipopeptides, glycolipids, GPI and glycoinositol-phospholipids, necrotic cells
PGRP-S	activation of PO cascade in insects activation of Toll in insects amidase activity in insects killing of Gram+ bacteria in PMNs killing or inhibition of growth of bacteria	Polysaccharides?
PGRP-L	induction of antibacterial peptides through *imd* pathway in insects phagocytosis of bacteria in insects activation of PO cascade in insects	polysaccharides?
PGRP-Iα	?	polysaccharides?
PGRP-Iβ	?	?
Nod1, Nod2	activation of NF-κB and pro-inflammatory mediators	MDP, muramyl peptides
Lysozyme	hydrolysis of PGN	none
Amidase	hydrolysis of PGN	none

* In mammals, unless otherwise indicated. See text for references.

How can a single molecule (CD14) bind with high affinity to such a wide variety of molecules? Mapping and comparing the LPS and PGN binding sites on CD14 suggest that these binding sites are conformational, rather than linear, and that they are only partially identical and partially different. It seems that the PGN and LPS binding sites on CD14 are formed by one common sequence (amino acids 51–64) and by other sequences that are different for LPS (amino acids 7–14 and 33–44) and PGN (amino acids 135–146) [17]. Therefore, it appears that such a usage of different regions of CD14 gives this molecule its amazing ability to interact with such a great variety of different ligands. It should also be noted that although leucine-rich repeats are generally considered as versatile binding motifs [10], in CD14, only two of the above-mentioned regions coincide with the LRRs (amino acids 7–14 and 135–146), and the remaining two (amino acids 33–44 and 51–64) are located outside the LRRs [17–19].

The exact PGN structure recognized by CD14 is not known. However, high molecular weight polymeric PGN is required for both CD14-mediated cell activation and for binding to CD14 [14, 15, 17]. Low molecular weight soluble PGN fragments, such as muramyl dipeptides, disaccharide dipeptides or pentapeptides do not bind to CD14, do not inhibit binding of high molecular weight polymeric PGN to CD14 and do not activate cells through CD14 [14, 17]. However, synthetic muramyl dipeptide immobilized on agarose (to resemble polymeric PGN) does bind CD14 [17].

Soluble CD14 forms complexes with LPS, and these complexes activate membrane CD14-negative cells, such as vascular endothelial cells, epithelial cells, vascular smooth muscle cells, fibroblasts and astrocytes [20, 21]. By contrast, the complexes of soluble CD14 with PGN do not activate CD14-negative cells [22]. Another function of soluble CD14 is to enhance the responses of CD14-positive cells. Indeed, the responses of CD14-positive cells to both LPS and PGN are enhanced by soluble CD14 [23].

CD14-mediated responses to LPS are greatly enhanced by another protein present in normal serum, LPS-binding protein (LBP), which catalytically transfers single LPS molecules from LPS aggregates (that are normally present in an aqueous environment) onto CD14 [18, 19, 24]. The CD14-mediated cell activation by PGN, however, is not enhanced by LBP, and this may be one reason why much higher concentrations of PGN than of LPS are needed to activate cells [12–15]. However, this may not be the only reason, because the difference in the affinity of binding of CD14 to LPS and PGN in the presence of LBP (K_D, 7 nM vs. 20 nM) is much smaller than the ~200 times difference between the effective macrophage-activating molar concentrations of LPS and PGN [17]. Therefore, other reasons are also likely to be responsible for this difference, such as, e.g. the effectiveness of transfer of the stimulant from CD14 to the cell-activating coreceptor (e.g. TLR, see below), or the effectiveness of interaction of the stimulant with the coreceptor.

Although CD14 is required for the activation of several cell types (especially monocytes) by LPS, PGN and other microbial macrophage activators, CD14 by itself most likely is not a fully functional signal-transducing cell-activating receptor. CD14 most likely only functions as a ligand-binding coreceptor. Based on the following several pieces of evidence, it was long suspected that another molecule (or receptor) is also involved in cell activation by PGN (and other activators). First, CD14 by itself cannot transmit the activating signal into the cell because CD14 is a GPI-linked molecule that does not have any transmembrane and cytoplasmic domains. Second, studies with LPS partial structures (that act as agonists or antagonists) indicate that CD14 by itself cannot always discriminate between agonistic and antagonistic structures [25]. Third, when cells of myeloid origin express CD14, they are responsive to PGN [12–15]. However, when cells of non-myeloid origin express CD14, they are unresponsive to PGN [22], which suggests that non-myeloid cells are missing some essential component(s) needed for the CD14-mediated response (such as a coreceptor or a signal-transducing molecule). Fourth, CD14 knockout mice are more than 1000 times less sensitive to LPS than the wild-type mice in several, but not in all LPS-induced responses [26, 27], and cells from these CD14 knockout mice are only 5–10 times less sensitive to PGN than the wild-type cells [28]. These findings again point to a possibility of CD14-independent responses to PGN and LPS.

Indeed, as discussed in the next section, most CD14-induced responses are mediated through TLRs. Whereas CD14 is required for the responses to LPS, which are mediated by TLR4, the TLR2-mediated responses to PGN do not require CD14, but are usually enhanced by CD14 (see next section). As also discussed below, interaction of bacteria or PGN with the CD14/TLR2 receptor system induces numerous pro-inflammatory host responses [7, 18, 19].

TLR2

TLRs are type I transmembrane molecules with an extracellular domain containing leucine-rich repeats and cytoplasmic TIR domain (Toll/IL-1 receptor), homologous to the IL-1 receptor. They were first discovered in *Drosophila melanogaster* as molecules required for dorso-ventral patterning in embryogenesis [3, 5, 29–31]. It was then realized that some members of the Toll receptor family also played a role in immunity in insects, because mutations in *toll* genes made flies more susceptible to infections [5, 29, 31]. Discovery of *Drosophila* Tolls led to the discovery and cloning of human TLRs [32, 33]. Because both overexpression of TLRs and cell stimulation by LPS activate the same signal transduction pathway that results in the activation of nuclear factor kappa B (NF-κB) [32, 33], it was then shown that transfection of LPS-unresponsive cells with TLR2 made these cells responsive to LPS [34, 35]. These results indicated that TLR2 may function as the long-sought signal transducing receptor for LPS. Concurrent positional cloning of the *lps* gene from LPS-unresponsive C3H/HeJ mice, identified TLR4 as the *lps* gene and the LPS receptor [36, 37]. It was later shown that the initially observed TLR2-mediated activation by LPS was mainly due to the contamination of LPS with LPS-associated lipoproteins [38, 39]. Thus, it is now firmly established that TLR4 is the primary receptor for LPS from enterobacteria, but for its receptor function TLR4 requires at least two other molecules, CD14 and MD-2 [3, 31, 40, 41].

Soon after the discovery of TLRs in humans, it was shown that TLR2 could function as the cell-activating receptor for Gram-positive bacteria and their PGN and LTA components [42, 43]. The function of TLR2 as the receptor for PGN, LTA and Gram-positive bacteria was further proven using TLR2 and TLR4 knockout mice [44], and confirmed by other reports [45–48]. It is now clear that TLR2 functions as a cell-activating receptor not only for Gram-positive bacteria, PGN and LTA, but also for lipoproteins, lipopeptides, mycobacterial lipoarabinomannan and fungal cell walls (zymosan) [3, 31, 45–54]. TLR2 may even function as a receptor for glycolipids, glycoinositol-phospholipids and necrotic cells [55, 56]. TLR2-mediated responses to PGN and other bacterial components usually do not require CD14, but are often enhanced by CD14 [39, 42–54].

Humans have 10 TLR receptors, and different TLRs are specific for different microbial products [2, 3, 31]. Different TLRs can form homo- or heterodimers, and the cell-activating function of TLR2 is dependent on the formation of heterodimers with TLR1 or TLR6 (fig. 2) [3, 31, 46]. Interestingly, although both TLR2 : TLR1 and TLR2 : TLR6 heterodimers function as PGN receptors, they may not function equally well as the receptors for other microbial products. For example, TLR2:TLR1 heterodimers preferentially function as the receptors for triacylated lipopeptides, and TLR2 : TLR6 heterodimers preferentially function as the receptors for diacylated lipopeptides [57, 58].

Insects have nine Toll receptors, but only one of them, the original Toll, functions in antimicrobial immunity [5]. However, in contrast to insect Toll, which does not directly interact with microbial products [5], mammalian TLRs most likely directly interact with microbial products [3, 31]. Recent studies suggest that PGN directly binds to TLR2 [59]. Cell activation studies indicate that a sequence of 25 amino acids (Ser^{40}–Ile^{64}) in the extracellular domain of TLR2 is required for the PGN-induced cell activation [60], thus suggesting that this sequence may constitute (or be a part of) the TLR2 binding site for PGN.

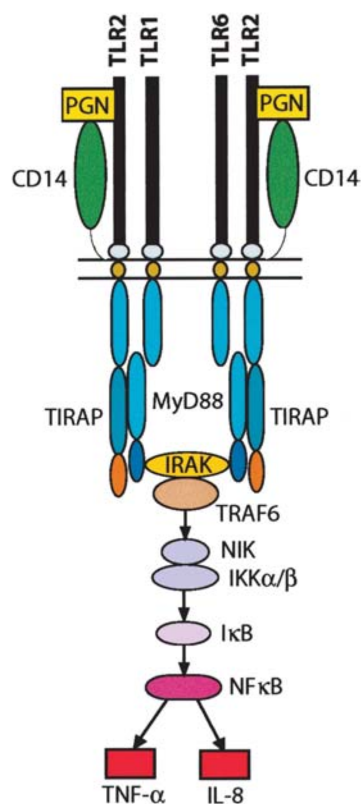

Figure 2. CD14/TLR2-mediated recognition of PGN. PGN is recognized by CD14 and complexes of TLR2 with TLR1 or TLR6. Engagement of TLRs results in MyD88-dependent activation of NF-κB, which is required for the activation of transcription cytokine and chemokine genes.

The exact PGN structure recognized by TLR2 is not known. However, it is likely similar to the structure recognized by CD14, because both CD14- and TLR2-mediated cell activation require high molecular weight polymeric PGN, and low-molecular weight soluble PGN fragments do not activate cells through TLR2 or CD14 [14, 15, 17, 42–47, 61]. Highly effective monocyte activation by long (crosslinked and branched) glycan-free PGN stem peptides was also shown [62], but it is not known whether this activation is TLR2- and CD14-mediated. Moreover, this activation has not been yet confirmed with synthetic peptides.

TLR2 is primarily expressed on monocytes, macrophages, dendritic cells, B cells and, to a lesser extent, on neutrophils and a few other cells [63]. The main consequence of interaction of PGN with TLR2 is activation of a signal transduction pathway that results in the activation of NF-κB, which is required for the activation of transcription of several cytokine and chemokine genes, such as tumor necrosis factor-α (TNF-α) and IL-8 (fig. 2) [2, 3, 31, 42, 43, 61, 64]. Other signal transduction pathways are also activated [65, 66]. TLR activation results in the induction of secretion of numerous chemokines and cytokines and other mediators of inflammation [2, 3, 31, 61, 64–66].

The combined effects of numerous mediators of inflammation (several of which are TLR2-induced cytokines and chemokines) on the host in vivo are at least partially responsible for the numerous biologic effects of PGN. These biologic effects mimic several clinical manifestations of bacterial infections, such as inflammation, pus formation, leukocytosis, fever, acute-phase response, hypotension, sleepiness, decreased appetite, arthritis and immune adjuvant activity [7, 8, 18, 19, 67]. The effects induced by different bacterial products through different TLRs are similar, but not identical. There is a set of common responses induced by most TLRs, and a number of responses unique for each ligand and its TLR [2, 3, 31, 66]. Likewise, the in vivo biological effects of various TLR agonists are similar, but not identical. For example, LPS is highly toxic in vivo and easily induces shock and death, whereas PGN is not toxic and does not induce shock. On the other hand, PGN is highly arthritogenic, and LPS is not [7, 8, 18, 19, 67]. However, the in vivo biologic effects of microbial products usually result from complex interactions of these compounds with various host defense mechanisms and often cannot be attributed to a single receptor.

Activation of TLR2 also has an adjuvant effect through induction of dendritic cell maturation and initiation of the acquired immune response to antigens [3, 31, 66]. It is interesting to note that although the adjuvant effect of PGN has been known for 3 decades [7, 67, 68], only now do we realize that TLRs are needed for the initiation of acquired immune responses, and in particular for the activation of antigen-presenting cells and induction of expression of costimulatory molecules on these cells [3, 31, 66]. Thus, the discrimination between self and non-self is routinely done by the antigen-presenting cells through TLRs. PGN's adjuvant effect is also likely due to the recognition of PGN-derived fragments (muramyl peptides) by Nod proteins (see below).

PGRPs

Peptidoglycan recognition proteins (PGRPs) are another recently identified family of innate immunity pattern recognition molecules that are highly conserved from insects to mammals [69–71]. The first member of the PGRP family, now designated PGRP-S (for 19-kDa PGRP-short), was discovered in 1996 as a protein present in the hemolymph and cuticle of a silkworm (*Bombyx mori*) [72]. It binds Gram-positive bacteria and PGN and activates the prophenoloxidase cascade [72]. Prophenoloxidase cascade is an innate immunity mechanism in insects that generates antimicrobial products, surrounds microorganisms with melanin and contains the infection

[72, 73]. PGRP-S was then identified and cloned in a moth (*Trichoplusia ni*) as a protein that is upregulated by a bacterial challenge [69]. Subsequently, PGRP-S from a silkworm (*B. mori*) was also cloned [74].

Sequencing of the *Drosophila* genome has led to the discovery of a family of 13 highly diversified PGRP homologues [70, 75]. Based on the predicted structures of the gene products, *Drosophila* PGRPs were grouped into two classes: short PGRPs (PGRP-S), which are small extracellular proteins similar to the original PGRP, and long PGRPs (PGRP-L), which have long transcripts and are either intracellular or membrane-spanning proteins (fig. 3). Recent sequencing of the mosquito (*Anopheles gambiae*) genome enabled identification of seven PGRP genes: three of these genes code for three short PGRPs and four of these genes code for seven long PGRPs, some of which are splice variants (fig. 3) [75].

Many of the insect PGRPs are expressed in immune competent organs, such as the fat body, gut and hemocytes, and their expression is upregulated by injections of PGN and bacteria [69, 70, 74–76], suggesting their role in insect immunity. Indeed, all so far identified functions of insect PGRPs are important for the antimicrobial innate immunity.

As mentioned above, moth PGRP-S recognizes PGN and Gram-positive bacteria and activates the prophenoloxidase cascade [72, 73]. *Drosophila* PGRP-SA is required for the activation of Toll receptor pathway by Gram-positive bacteria [77]. The activation of Toll, however, is indirect and requires proteolytic cleavage of Spaetzle, an extracellular cytokine-like protein present in insect hemolymph that serves as an endogenous activator of *Drosophila* Toll [5, 77]. Proteolytic cleavage of Spaetzle can also be induced by another pathway that is initiated by fungi [5, 78]. Activation of *Drosophila* Toll results in the induction of antibacterial and antifungal peptides and generation of an effective immunity to Gram-positive bacteria and fungi [5, 77, 78].

Drosophila PGRP-LC is predicted to be a transmembrane protein (fig. 3), and it may function as a cell-surface receptor (or coreceptor) to activate the '*imd*' pathway (named after 'immune deficient' mutants) in re-

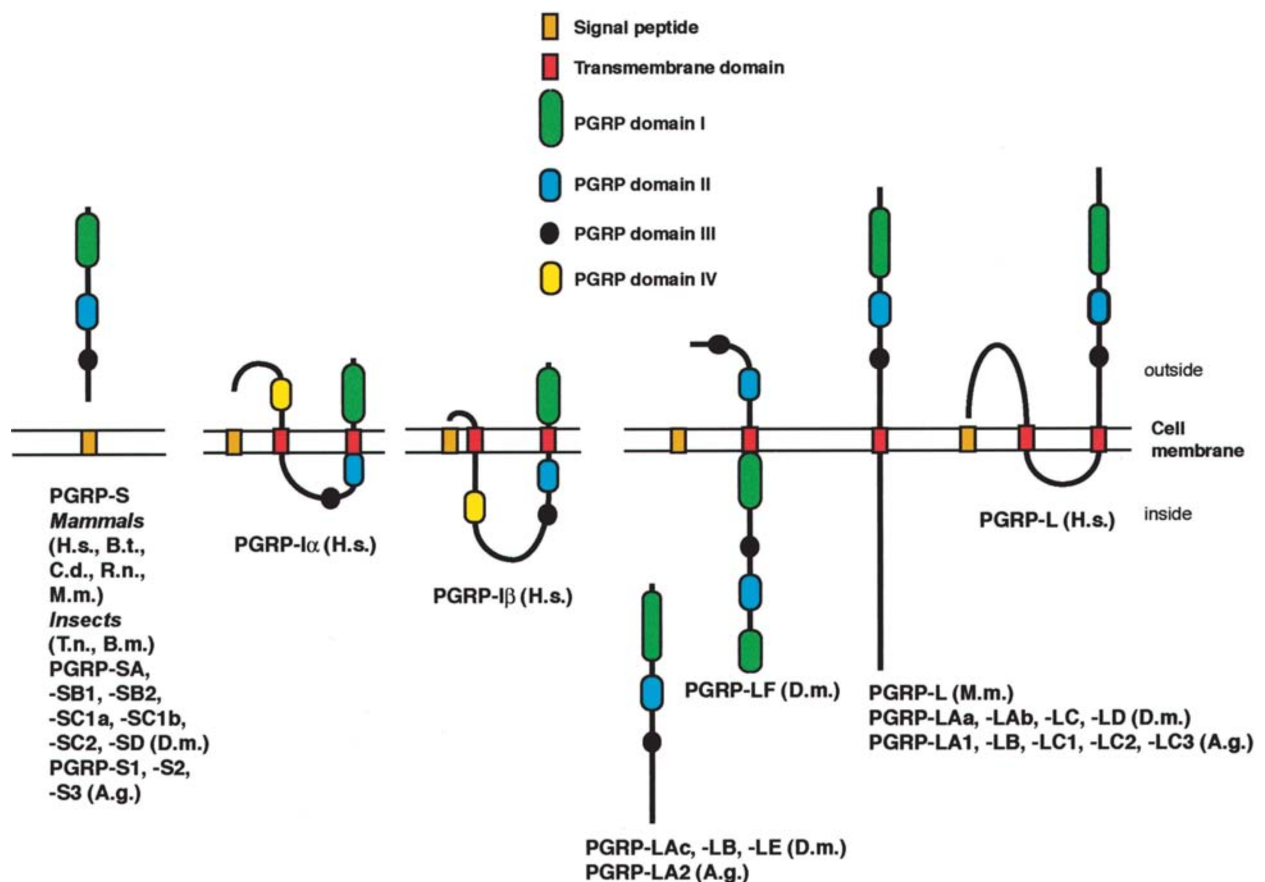

Figure 3. Predicted domains and cellular locations of mammalian and insect PGRP proteins. The protein sequences of most of the PGRPs were deduced from complementary DNA (messenger RNA) or expressed sequence tags and the domains and cellular locations were predicted by computer modeling (the GenBank accession numbers are listed in the references) [69–71, 75, 76]. Mammals: B.t., *Bos taurus*; C.d., *Camelus dromedarius*; H.s., *Homo sapiens*; M.m., *Mus musculus*; R.n., *Rattus norvegicus*. Insects: A.g., *Anopheles gambiae*; B.m., *Bombyx mori*; D.m., *Drosophila melanogaster*; T.n., *Trichoplusia ni.*

sponse to Gram-negative [79–81] and some Gram-positive bacteria [80]. This pathway is somewhat similar to the mammalian TNF-α receptor-induced pathway and in *Drosophila* results in the production of antibacterial peptides with the activity primarily against Gram-negative bacteria [79, 80]. The specificity of *Drosophila* PGRP-LC for Gram-negative bacteria may not be absolute, because one study indicates that PGRP-LC also mediates responses against Gram-positive bacteria and PGN from Gram-positive bacteria [80]. Moreover, *Drosophila* PGRP-LC also participates in the phagocytosis of bacteria [81]. *Drosophila* PGRP-LE was also recently reported to activate both the *imd* pathway and the prophenoloxidase cascade [82]. It is not certain, however, how these two functions are accomplished, because it is not known whether the location of PGRP-LE is intracellular or extracellular. PGRP-LE does not have a transmembrane domain and does not have a signal peptide, and thus it is predicted either to have an intracellular location or to be released from the cells by an unknown mechanism [82].

Cloning of insect PGRP-S has led to the discovery and cloning of mouse and human [69] and, subsequently, rat, camel and bovine PGRP-S orthologues [83, 84]. Sequencing of the human genome allowed the discovery and cloning of three additional human PGRPs, designated PGRP-L, PGRP-Iα and PGRP-Iβ [71], which together with PGRP-S form a family of four human PGRPs (fig. 3). Human PGRP-L, PGRP-Iα and PGRP-Iβ all have two predicted transmembrane domains [71], in contrast to mouse PGRP-L and several insect PGRP-L, which have one predicted transmembrane domain (fig. 3).

Mammalian PGRPs have highly conserved C termini that contain PGRP domains (fig. 3). PGRP-S from all five mammalian species also have six conserved cysteines that are likely to form three disulfides. The presence of these three disulfides has been proven experimentally for the bovine PGRP-S orthologue [84]. These disulfides are likely to stabilize PGRP-S conformation in the harsh environment of leukocyte granules or extracellular conditions. Human PGRP-Iα and PGRP-Iβ also have these six conserved cysteines, whereas only four of these conserved cysteines are present in both mouse and human PGRP-L [71]. Therefore, at least two disulfides may be also present in these transmembrane PGRPs, but their actual presence needs to be confirmed experimentally.

Alignment of amino acid sequences of mammalian and insect PGRPs reveals high homology in the C terminal portion of the molecules [71]. PGRP domain II is especially highly conserved between mammals and insects (has 69% conserved identities and 83% conserved similarities) [71]. This C-terminal region, and especially PGRP domain II, is also highly homologous to the T7 bacteriophage lysozyme, which is an amidase (i.e. hydrolyzes the bond between muramic acid and the peptide in PGN [85]). PGRPs domain II has 45% conserved identities and 55% conserved similarities with lysozyme T7. Because of this homology, at least one PGRP (*Drosophila* PGRP-SC1B) also has amidase activity [86].

Two cysteines are conserved between mammalian and insect PGRPs. These cysteines seem to be essential for the function or structure of PGRPs, because a mutation in one of these cysteines in *Drosophila* PGRP-SA (C80Y) makes the molecule totally inactive [77]. Another cysteine (C168) is essential for the amidase activity of *Drosophila* PGRP-SC1B, because its mutation causes the loss of its amidase activity [86].

The N-terminal portions of PGRP molecules have very little homology within the PGRP family and between insects and mammals, and they also have no homology to any other known proteins [71]. They also lack easily identifiable functional motifs.

Phylogenetic analysis of mammalian and insect PGRPs shows that mammalian PGRP-Ls and PGRP-Iα and -Iβ form two separate branches that are unrelated to each other and are unrelated to insect PGRP-Ls [71]. Therefore, there are no insect orthologues of mammalian PGRP-L and PGRP-I, and mammalian and insect PGRP-Ls did not evolve from common ancestors. PGRP-Iα and -Iβ seem to have evolved from common ancestors of mammalian PGRP-S [71].

Human PGRPs have highly differential expression in various organs and tissues. PGRP-S is highly expressed in the bone marrow (and to a lower extent in neutrophils and fetal liver), PGRP-L is highly expressed in the liver, and PGRP-Iα and PGRP-Iβ in the esophagus, and to a lower extent in tonsils and thymus [71]. Therefore, different mammalian PGRPs are likely to perform different functions unique to these organs.

Indeed, mammalian PGRP-S is stored in the PMN's specific (gelatinase) granules [87], is bacteriostatic [87, 88] and functions in intracellular killing of bacteria in PMNs [87]. Consequently, PGRP-S-deficient (*PGRP-S*$^{-/-}$) mice have increased susceptibility to intraperitoneal infection with Gram-positive bacteria of low virulence, but not with more virulent Gram-positive or Gram-negative bacteria [87]. Neutrophils from *PGRP-S*$^{-/-}$ mice have normal phagocytic uptake of bacteria, but are defective in intracellular killing and digestion of Gram-positive bacteria of low virulence [87]. Bovine PGRP-S orthologue is also present in PMN granules and has antibacterial activity [84]. Therefore, mammalian PGRP-S functions as an antibacterial neutrophil granule protein. Unlike *Drosophila* PGRP-SA, mouse PGRP-S does not interact with the TLR/CD14 cell activation system, and TLR2/CD14-mediated induction of cytokines in *PGRP-S*$^{-/-}$ mice is normal [87]. Moreover, unlike *Drosophila* PGRP-SC1B, mouse PGRP-S has no amidase activity [69, 86, 88]. Thus, the effector functions of PGRP-S in mammals and

insects are different, and only the bacterial recognition function of PGRP-S, but not its effector function, is conserved in evolution from insects to mammals.

All PGRPs tested so far (insect PGRP-S and mouse and human PGRP-S, -L and Iα) bind PGN and bacteria [69–72, 86]. However, although PGRPs were originally described as PGN-binding and recognition proteins [69–72], it is now becoming clear that the specificity of at least some PGRPs is likely to be broader than just for PGN or Gram-positive bacteria. Whereas *B. mori* PGRP-S and *D. melanogaster* PGRP-SA recognize only Gram-positive bacteria [72, 77], *D. melanogaster* PGRP-LC recognizes Gram-negative bacteria, in which PGN is located under the outer membrane and, thus, not easily accessible on the bacterial surface [79–81]. *D. melanogaster* PGRP-LC may also recognize PGN and Gram-positive bacteria [80]. Bovine PGRP-S orthologue (named OBP for oligosaccharide-binding protein) is bactericidal or bacteriostatic for both Gram-positive and Gram-negative bacteria and even fungi (which lack PGN) [84], and binds to LPS as well as or better than to PGN [89]. Also, human PGRP-S, -L and -Iα all bind to both Gram-positive and Gram-negative bacteria and some fungi, although each of these PGRPs preferentially binds to different bacteria and fungi [R. Dziarski and Z.-M. Wang, unpublished]. The identities of the molecules on Gram-positive and Gram-negative bacteria and fungi, to which these PGRPs bind, are currently unknown, but are likely to be polysaccharide determinants found only in some bacteria or fungi because of different preferential binding of each PGRP to different bacteria or fungi. Moreover, at least one PGRP (human PGRP-Iβ) binds very poorly to bacteria and fungi, and thus is likely to be specific for a so far unidentified (possibly nonbacterial) product [71 and R. Dziarski and Z.-M. Wang, unpublished]. However, it should be also noted that at least some PGRPs, e. g. mouse PGRP-S, are highly specific for PGN because their affinity of binding to PGN is 13 nM and their affinity of binding to other bacterial cell wall components, such as LPS or LTA, is ~1000 times lower [88].

Binding of PGN and bacteria to mammalian transmembrane PGRPs suggests their direct role in recognition of bacteria. However, the consequences of this binding and, thus, the exact functions of PGRP-L and PGRP-Iα and -Iβ in innate immunity to bacteria in mammals are not known.

Nod

Nods are a family of cytoplasmic proteins with structural homology to a large family of plant R (resistance) proteins. In mammals, they include Nod1, Nod2 and several other homologues [90]. They have a C-terminal domain containing leucine-rich repeats, nucleotide-binding oligomerization domain (NOD) and an N-terminal CARD (caspase-recruitment) domain [90]. They likely function as intracellular regulators of cell activation [90]. Nod1 has ubiquitous expression in several tissues and cell types, and expression of Nod2 is restricted to monocytes [90, 91]. Nod1 and Nod2 mediate activation of NF-κB through association with a serine-threonine kinase, RICK [91–93]. This activation is TLR- and MyD88-independent and thus serves as an alternative pro-inflammatory pathway. The significance of Nods is underscored by the association of mutations in Nod2 with increased susceptibility to Crohn's disease [94, 95] and Blau syndrome [96].

LPS and intracellular LPS-containing Gram-negative bacteria were first identified as the activators of Nod1 and Nod2 [93, 97]. However, the most recent results indicate that the actual activators for Nod 2 [98, 99], and also Nod 1 [100], and are low molecular weight PGN fragments, including a synthetic PGN fragment, muramyl dipeptide (*N*-acetylmuramyl-L-alanyl-D-isoglutamine, MDP). Nod1 and Nod2 are unresponsive to purified LPS, and it appears that the originally reported responsiveness of Nod1 and Nod2 to LPS was due to contamination of the LPS preparations with PGN fragments (muramyl peptides). Nod1 and Nod2 seem to be unresponsive to high molecular weight (undigested) polymeric PGN [98], which confirms previously reported unresponsiveness or low responsiveness of Nod1 and Nod2 to polymeric PGN [97].

It is not known whether Nods directly interact with PGN fragments and muramyl peptides. However, transfection with Nods is sufficient to convert MDP-unresponsive cells into MDP-responsive cells, and Nods can discriminate between active MDP (containing L-alanyl-D-isoglutamine) and inactive MDP stereoisomers (containing L-alanyl-L-isoglutamine or D-alanyl-D-isoglutamine) [98, 99]. Also, the mutated Nod2 that is associated with the susceptibility to Crohn's disease [94, 95] is unresponsive to muramyl peptides and PGN fragments [98, 99], but is still able to induce some constitutive NF-κB activation. Thus, the PGN-recognition function of Nod2 is highly specific for the L-D amino acid configuration present in MDP and PGN, and the recognition function of Nod2 can be separated from its signaling function. It is also worth noting that MDP is a synthetic analogue of the minimal common structure usually present in most PGN from both Gram-positive and Gram-negative bacteria, and that MDP was first synthesized as the minimal structure responsible for the adjuvant activity of PGN [67, 68, 101]. However, MDP is not a natural fragment, and it is not generated in vivo by enzymatic digestion of PGN [8, 62, 67, 68]. Nevertheless, mammalian cells have a system to specifically recognize MDP as the common structural motif present in most PGN [6, 7].

Activation of Nod1 and Nod2 by muramyl peptides explains the mechanism of adjuvant activity of MDP and related muramyl peptides [67, 68, 101], which until now has been poorly understood, and confirms the previously shown CD14 and TLR independence of cell activation by MDP [17, 102]. As mentioned earlier, CD14 and TLR2 are unresponsive to MDP, and they require high molecular weight polymeric PGN or larger PGN fragments for activation [17, 102]. Therefore, activation of Nods by muramyl peptides also explains the mechanism of synergism between MDP (which works through Nods) and LPS [103–106] or PGN [106] (which work through CD14 and TLRs), a phenomenon which until now has also been poorly understood.

Note added in proof. Recent results (reported after submission of this paper) indicate that the bacterial component that is recognized by *D. melanogaster* PGRP-LC and activates the *imd* pathway is the m-Dap-containing PGN uniquely present in all Gram-negative bacteria and in some Gram-positive bacteria (mainly genus *Bacillus* and *Clostridium*) [107]. The bacterial component that is preferentially recognized by *D. melanogaster* PGRP-SA and initiates activation of the Toll receptor is the Lys-containing PGN present in Gram-positive cocci, although the m-Dap-containing PGN also weakly activates the Toll receptor [107]. Interestingly, the mammalian Nod1 is also specific for the m-Dap-containing PGN [100], whereas Nod2 is specific for MDP present in all PGN [98, 99].

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