

NOD-LRR PROTEINS: Role in Host-Microbial Interactions and Inflammatory Disease

Naohiro Inohara, Mathias Chamillard,
Christine McDonald, and Gabriel Nuñez

Department of Pathology and Comprehensive Cancer Center, The University of Michigan Medical School, Ann Arbor, Michigan 48109; email: ino@umich.edu, mathiasc@umich.edu, mcdonalc@umich.edu, bclx@umich.edu

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■ **Abstract** Nods are cytosolic proteins that contain a nucleotide-binding oligomerization domain (NOD). These proteins include key regulators of apoptosis and pathogen resistance in mammals and plants. A large number of Nods contain leucine-rich repeats (LRRs), hence referred to as NOD-LRR proteins. Genetic variation in several NOD-LRR proteins, including human Nod2, Cryopyrin, and CIITA, as well as mouse Naip5, is associated with inflammatory disease or increased susceptibility to microbial infections. Nod1, Nod2, Cryopyrin, and Ipaf have been implicated in protective immune responses against pathogens. Together with Toll-like receptors, Nod1 and Nod2 appear to play important roles in innate and acquired immunity as sensors of bacterial components. Specifically, Nod1 and Nod2 participate in the signaling events triggered by host recognition of specific motifs in bacterial peptidoglycan and, upon activation, induce the production of proinflammatory mediators. Naip5 is involved in host resistance to *Legionella pneumophila* through cell autonomous mechanisms, whereas CIITA plays a critical role in antigen presentation and development of antigen-specific T lymphocytes. Thus, NOD-LRR proteins appear to be involved in a diverse array of processes required for host immune reactions against pathogens.

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INTRODUCTION

Elimination of infectious agents by the host plays a critical role in the survival of metazoans. Understanding how host organisms detect invasive pathogens and elicit a defense response, leading to the clearance of the infectious agents, has been a long-standing question for immunologists. The problem is intriguing because pathogens comprise a large group of agents with enormous molecular diversity and high mutation rates. In response to pathogen infiltration, the vertebrate immune system has evolved multiple defense systems, which can be broadly separated into innate and adaptive immunity, to repel and kill the invasive microbe. The innate immune response exists in all multicellular organisms and is primarily mediated by professional immunocytes that are capable of engulfing and destroying a broad range of pathogens (1). During the first days of a primary infection, the innate immune system plays a critical role in development of adaptive immunity, which is mediated by T and B lymphocytes, whose hallmarks are memory and specificity.

Initial sensing of microbial agents by the host is mediated by the recognition of pathogen-associated molecular patterns (PAMPs), which are highly conserved structures expressed uniquely by microbes of the same class. PAMPs are recognized by specific host pattern-recognition molecules (PRMs) (1). One class of PRMs (e.g., lectin proteins) binds to native or opsonized pathogens at extracellular sites and mediates their elimination through recruitment of antimicrobial factors, such as complement molecules. Another class of PRMs is involved in induction of microbicidal host responses through intracellular signaling events. The latter group of PRMs includes Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-containing proteins (Nods). The importance of these factors in innate immunity was initially discovered in fruit flies and/or plants, which lack classical adaptive immune systems (2, 3).

CONSERVATION OF PATHOGEN DEFENSE SYSTEMS IN ANIMALS AND PLANTS

First identified in the fruit fly, TLRs play a pivotal role in mediating defense responses to fungal, bacterial, and viral pathogens (1, 3, 4). TLRs are transmembrane proteins that contain leucine-rich repeats (LRRs) in their ectodomains for PAMP recognition. TLR signaling is mediated through a conserved cytosolic module, called the Toll/interleukin-1 receptor (TIR) domain. The intracellular TIR domain of TLRs plays an essential role in transducing TLR signaling events by interacting with cytosolic factors (1, 4). The cellular response, induced through TLR signaling, mediates innate immune responses and, in vertebrates, stimulates adaptive immunity, which leads to the eventual eradication of the invading pathogen. Host defense initiated by TLRs stimulates phagocytes via the secretion of intercellular mediators, including proinflammatory cytokines and chemokines, and promotes infiltration of a robust adaptive response via the induction of cell surface costimulatory

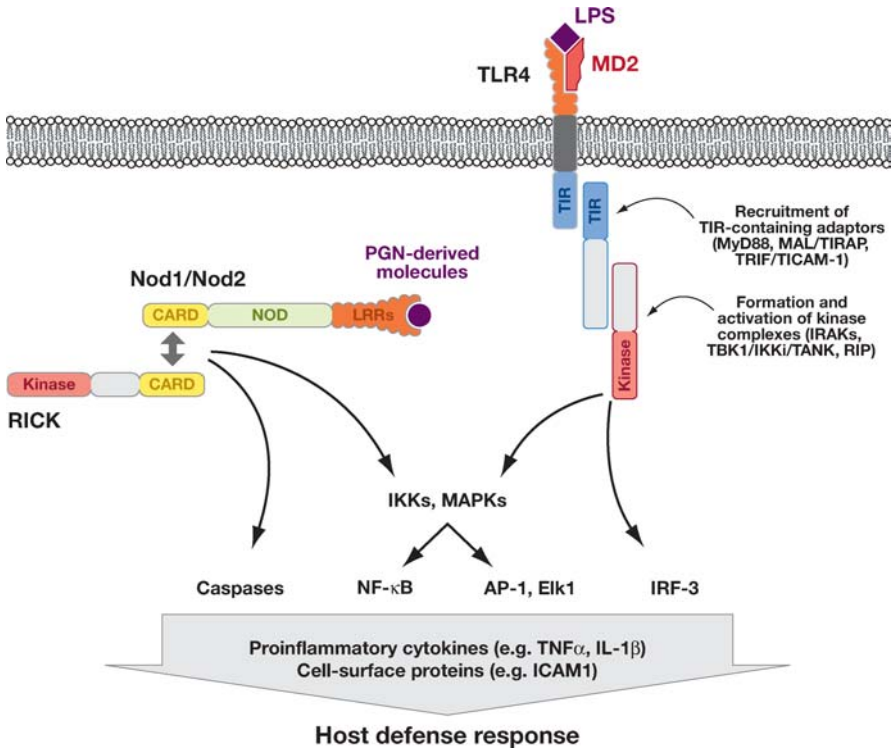


Figure 1 Nod1, Nod2, and TLR4 signaling pathways. In this schematic overview, Nod1 and Nod2 are involved in the recognition of bacterial peptidoglycan (PGN)-derived molecules, resulting in recruitment of a downstream kinase protein, RICK/RIP2/RIPK2/CARDIAK, through CARD-CARD interaction. Once RICK is recruited, Nod1 and Nod2 activate transcriptional factors (NF- κ B, AP-1, and Elk-1) and caspases. TLR4 complexed with MD2 is essential for the recognition of lipopolysaccharide (LPS) at the cell surface, resulting in recruitment of several downstream factors, including TIR-containing adaptors (e.g., MyD88, MAL/TIRAP, TRIF/TICAM-1). Once these downstream factors are recruited, several kinase complexes (IRAKs, TBK1/IKKi/TANK and RIP) are formed and activated which result in IKKs, MAPKs, and IRF-3 phosphorylation. These signaling events lead to the production of proinflammatory cytokines and cell-surface molecules (see details in the main text).

molecules in T cells. These cellular events are regulated at the transcriptional and/or post-transcriptional level through the activation of several protein kinases and transcriptional factors, including NF- κ B, AP-1, Elk-1, and IRFs (Figure 1) (1, 4).

Pathogen recognition systems are also found in plants. Recognition of pathogens in plants is mediated by disease resistance (*R*) genes that elicit the hypersensitivity response, which includes physical isolation of infected lesions through remodeling of cell wall structures, production of reactive oxygen species, cell death, and

production of antipathogen molecules at the site of infection (2). Some *R* gene products are transmembrane proteins with ectodomains containing LRRs, such as mammalian TLRs (5). Another major class of *R* gene products is the cytosolic Nod proteins, which typically contain amino-terminal α -helix-rich or TIR domains, a central NOD, and carboxyl-terminal LRRs (5). Some transmembrane plant *R* gene products (e.g., rice Xa21) contain protein kinase domains, which are homologous to a group of protein kinases (e.g., IRAKs, RICK, PKR, and TAK1) that are essential for innate immune responses mediated by TLRs, Nods, and other receptors (5). Therefore, plants and animals possess similar types of receptors and signaling molecules for pathogen recognition and innate host defense.

The observation that plants and animals contain transmembrane and cytosolic LRR-containing proteins for microbial immune recognition suggests that host cells can sense pathogens both at the cell surface and in the cytosol. Indeed, there is evidence that mammalian TLRs recognize PAMPs found in extracellular compartments (i.e., at the cell surface) or on the luminal side of intracellular vesicles (4, 6). Mammalian and plant host cells have also evolved surveillance mechanisms to recognize bacteria and other infectious microorganisms in the cytosol of the infected cell (5, 7, 8). There is evidence that the cytosolic bacterial recognition system mediates the induction of multiple signaling pathways, including NF- κ B activation, type I interferon production, and protein ubiquitination (Figure 1) (7, 9, 10). For example, host double-stranded RNA (dsRNA)-dependent protein kinase (PKR) and retinoid acid inducible gene I (RIG-I) helicase detect viruses through sensing the viral dsRNA produced during the infection cycle (11–13). However, the specific host mediators that detect and initiate these cytosolic immune responses against bacterial and/or viral agents are not well understood.

This review focuses on mammalian Nod proteins containing LRRs (referred to here as NOD-LRR proteins), a family of cytosolic proteins that has been recently implicated in innate recognition of bacteria and host defense. There is now evidence that two NOD-LRR proteins, namely Nod1 and Nod2, function as PRMs to induce signaling pathways upon recognition of bacterial PAMPs. Importantly, mutations in three human NOD-LRR proteins have been implicated in inflammatory diseases and/or immunodeficiency (14). In addition, genetic variation in Naip5, a mouse NOD-LRR protein, regulates the permissiveness of macrophages to *Legionella pneumophila* replication (15, 16). These findings indicate that NOD-LRR proteins play an important role in the regulation of multiple immune response aspects in mammals and plants.

A FAMILY OF NOD-LRR PROTEINS

Initial searches of genomic databases for proteins with homology to the apoptosis regulator, Apaf-1, and its nematode homolog, CED-4, revealed two related proteins, Nod1 (CARD4) and Nod2 (CARD15). Like Apaf-1 and CED-4, Nod1 and Nod2 contain amino-terminal caspase-recruitment domains (CARDs) linked to a

centrally placed NOD domain. But, unlike Apaf-1, Nod1 and Nod2 possess LRRs in their carboxyl termini. Subsequently, a large family of Apaf-1-related, NOD-containing molecules was identified that comprise >20 proteins in humans with a modular structure similar to Nod1 and Nod2 (Figure 2). These NOD-LRR proteins are also referred to as the CATERPILLER [CARD, transcription enhancer, R (purine)-binding, pyrin, lots of leucine repeats] family (17). Mammalian NOD-LRR proteins are structurally related to R proteins because they share the same modular structure. NOD-LRR proteins are expressed primarily in immune cells, although the expression of certain proteins, such as Nod1, is ubiquitous (Table 1). The majority of animal and plant NOD-LRR proteins are comprised of three distinct functional domains: an amino-terminal effector domain involved in signaling, a centrally located regulatory NOD domain, and carboxyl-terminal LRRs that serve as a ligand-recognition domain (Figure 2).

The effector domains of mammalian NOD-LRR proteins are structurally variable, linking these proteins to multiple signaling pathways and biological functions. These effector domains are involved in homophilic and heterophilic interactions with downstream signaling partners. The diversity of the effector domains allows NOD-LRR proteins to interact with a wide array of binding partners and to activate multiple signaling pathways (Table 1). Effector domains involved in homophilic association include the caspase-recruitment domain (CARD) (18) and the pyrin domain (PYD, also called DAPIN and PAAD) (19). Both the PYD and the CARD belong to the death domain-fold family characterized by six α -helices that are tightly packed and include the death domain (DD) and death effector domain (DED) (20). Only 3 human NOD-LRR proteins possess amino-terminal CARDS, whereas NOD-LRR proteins possessing a PYD are, by far, the most numerous and include 14 proteins designated NALPs (Table 1).

Several NOD-LRR proteins contain amino-terminal sequences that are not involved in homophilic protein interactions, including neuronal apoptosis inhibitor proteins (NAIPs) and CIITA (21, 22). Instead of a CARD or PYD, the amino termini of NAIPs are composed of amino-terminal baculovirus-inhibitor-of-apoptosis repeats (BIRs) (22). CIITA is a transcriptional coactivator involved in the regulation of major histocompatibility complex class (MHC) genes, especially class II (MHC-II) (21, 23). CIITA contains an amino-terminal transcriptional activation domain that is essential for MHC gene transactivation through its interaction with multiple nuclear factors, including CBP/p300, RFX5, NF-Y, and CREB (23). Other variations on the classical NOD-LRR protein structure are seen in certain family members. Some Nod proteins such as PYNOD lack the carboxyl-terminal LRRs and structurally resemble nematode CED-4 (24). Several NOD-LRR proteins in animals and plants exhibit a more complex domain structure. For example, in addition to the NOD and LRRs, NALP1 contains an amino-terminal PYD, a carboxyl-terminal domain homologous to a unique region (NC) found in CARDINAL/CARD8, a truncated PYD, and a CARD (Figure 2). Similarly, the *Arabidopsis thaliana* RRS1 protein is composed of two amino-terminal TIR domains and a carboxyl-terminal WRKY domain (25).

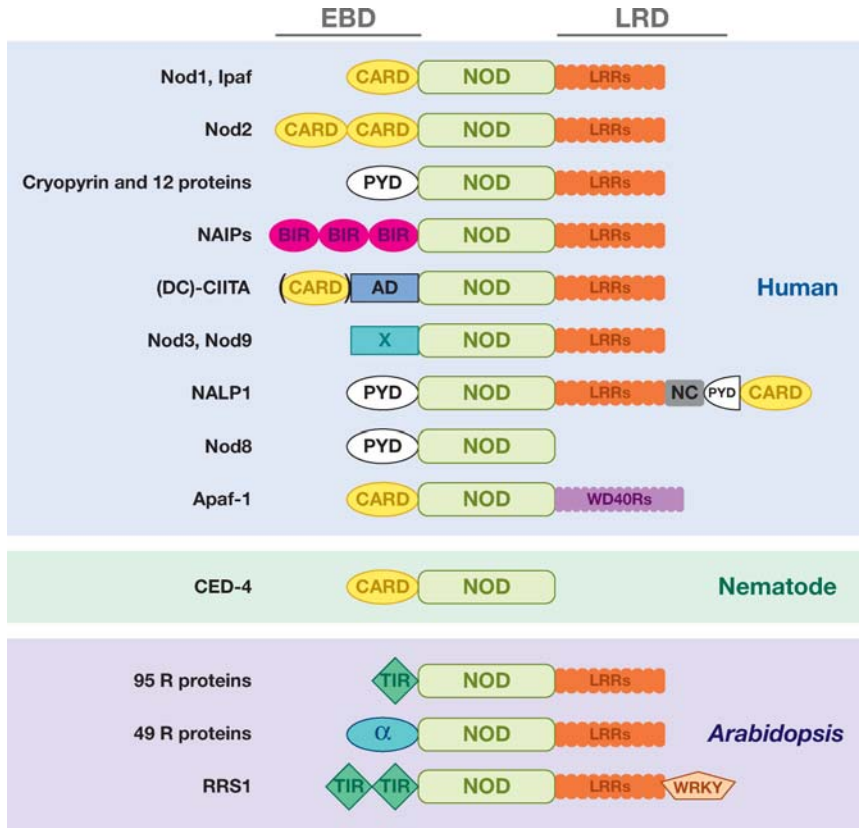


Figure 2 Domain structure of Nods. The majority of Nod proteins are composed of variable amino-terminal effector-binding domains (EBDs), a centrally located nucleotide-binding oligomerization domain (NOD) that mediates self-oligomerization, and a carboxyl-terminal ligand-recognition domain (LRD). The putative EBDs of NOD3 and NOD9 (represented by X) do not share significant homology with any known protein. The number of leucine-rich repeats (LRRs) varies in Nod proteins. The WRKY domain is a zinc finger-like domain found in plant W-box-binding transcription factors (25). The caspase-recruitment domain (CARD) shown in parenthesis is present in CIITA expressed in dendritic cells (166). An incomplete pyrin domain (PYD) is present in the carboxyl-terminal region of terminus of NALP1. Other abbreviations: α , α -helix/coiled-coil rich; NC, NALP1/CARDINAL expanded homology domain; WD40R, WD40 repeat; BIR, baculoviral inhibitor-of-apoptosis repeat; TIR, Toll interleukin-1 receptor domain.

TABLE 1 The human Nod protein family

Protein	Synonyms	Locus^a	EBD^b	Expression^c	LRD^d	Effectors^e	Ligand	Molecular function^e
Nod1	CARD4	7p15.1	CARD	Ubiquitous [I]	LRRs	RICK	iE-DAP	NF- κ B/caspase activation
Nod2	CARD15	16q12.1	CARDx2	APC [P]	LRRs	RICK	MDP	NF- κ B/caspase activation
Ipaf	CARD12, CLAN	2p22.3	CARD		LRRs	ASC		NF- κ B/caspase activation
Cryopyrin	NALP3, PYPAF1, CIAS1	1q44	PYD	APC [P]	LRRs	ASC		NF- κ B/caspase activation
PYPAF7	Monach1, NALP12, RNO2	19q13.42 ^f	PYD	APC	LRRs			NF- κ B/caspase activation
Nod12	PYPAF3, NALP7	19q13.42 ^f	PYD		LRRs			
NALP2	PYPAF2	19q13.42 ^f	PYD	Ubiquitous	LRRs			
Nod6	NALP9	19q13.42 ^f	PYD	Ubiquitous	LRRs			
Nod17	PYPAF6, NALP11	19q13.42 ^f	PYD	Ubiquitous	LRRs			
PAN2	PYPAF4, NALP4	19q13.42 ^f	PYD	Ubiquitous	LRRs			
Nod14	NALP13	19q13.43 ^f	PYD		LRRs			
Nod16	NALP8	19q13.43 ^f	PYD		LRRs			
Mater	PYPAF8, NALP5	19q13.43 ^f	PYD	E	LRRs			
PYPAF5	NALP6, Monach1	11p15.5	PYD	Ubiquitous	LRRs			
Nod5	NALP14	11p15.43	PYD	Ubiquitous	LRRs			
Nod27		16q13	PYD	APC	LRRs			
CIITA		16p13.13	AD	APC, B, T, NK [I, P]	LRRs	TFs		MHC-II induction

(Continued)

TABLE 1 (Continued)

Protein	Synonyms	Locus ^a	EBD ^b	Expression ^c	LRD ^d	Effectors ^e	Ligand	Molecular function ^e
Nod3		16p13.3	X	Ubiquitous	LRRs			
Nod9		11q23.3	X	Ubiquitous	LRRs			
NAIP	BIRC1	5q13	BIRx3	APC, mφ [P]	LRRs			
NALP1	NAC, DEFCAP, CARD7	17p13	PYD, CARD ^g	APC, B, T, NK	LRRs	ASC, caspases ^f		Caspase activation
PYNOD	NALP10, NOD8	11p15.4	PYD	Ubiquitous	None			
Apaf-1		12q23	CARD	Ubiquitous [P]	WDRs	Caspase-9	Cytochrome <i>c</i>	Caspase activation

^aLocus location according to University of California Santa Cruz, Genome Bioinformatics Site, <http://genome.ucsc.edu/>

^bAbbreviations are EBD, effector-binding domain; BIR, baculoviral IAP repeat domain; CARD, caspase recruitment domain; PYD, pyrin domain; AD, activation domain; and X, unclassified domain.

^cTissues with highest levels of gene expression according to SymAtlas v0.7.3 (<http://symatlas.gnf.org>) are shown. The stimuli that induce gene expression are shown in parenthesis. APC, antigen-presenting cells, and their precursors (BDCA4+, CD33+, or CD14+ cells); B, CD19+ B cells; mφ, macrophage; ECD71+ early erythroid; NK, CD56+ NK cells; T, T cells; I, IFNγ; P, proinflammatory molecules (including LPS, TNF, or IL-1).

^dAbbreviations are LRD, ligand-recognition domain; LRR, leucine-rich repeats; and WDR, WD40 repeats.

^eFor simplicity, only the function and downstream effector molecules that have been reported by two or more groups are shown. Abbreviation is TFs, transcription factors.

^fThese genes are tandemly located on 19q13.42-43 in the following order from the centromere: PYPAF7, Nod12, NALP2, Nod6, Nod17, PAN2, Nod14, Nod16 and Mater, PYPAF7 and Nod12 are separated by 0.9-Mbp region, containing about 40 genes, whereas NALP2 and Nod6 are separated by 0.7-Mbp region containing about 30 unrelated genes.

^gNALP1 has been reported to bind ASC through N-terminal PYD and to bind caspase-4 and -5 through carboxyl-terminal CARD.

SIGNALING PATHWAYS INDUCED THROUGH NOD-LRR PROTEINS

The amino-terminal region of mammalian NOD-LRR proteins mediates the binding to downstream molecules and is thought to be the major signaling domain (Table 1). For example, Nod1 and Nod2 physically associate with RICK (RIPK2/RIP2/CARDIAK), a CARD-containing protein kinase, through homophilic CARD-CARD interactions (26–28). Expression of Nod1, Nod2, or RICK induces NF- κ B activation (26–28). Mutational analyses demonstrate that the CARDS of Nod1 and Nod2 are necessary and sufficient for the activation of NF- κ B, implying that the CARDS of these Nod proteins function as effector domains (26–28). RICK is critical in these signaling pathways because Nod1 and Nod2 do not activate NF- κ B in mouse embryo fibroblasts derived from mice deficient in RICK, and activation is restored upon ectopic expression of RICK in the cells (29, 30).

An intermediate region located between its CARD and its kinase domain mediates RICK interaction with the regulatory subunit NEMO/IKK γ , linking Nod1 and Nod2 to the phosphorylation of I κ B- α and activation of NF- κ B (Figure 1) (31). NF- κ B activation induced through Nod1 is not blocked by dominant negative forms of MyD88 and TRAF6, molecules that are components of TLR signaling (26, 32). These results suggest that Nod1, Nod2, and TLRs induce NF- κ B by converging on activation of the IKK complex, but do so via divergent routes (Figure 1). Some studies suggest that RICK may also contribute to TLR signaling because macrophages deficient in RICK showed reduced signaling, induced by certain TLR ligands, including lipopolysaccharide (LPS) (30). By contrast, human peripheral blood mononuclear cells and mouse macrophages lacking functional Nod1 or Nod2 showed no obvious reduction of LPS signaling (33–38). Many PAMP preparations are contaminated with Nod1- and Nod2-stimulating molecules (33, 35, 39–41), and this could explain, at least in part, reduced TLR signaling observed in RICK-deficient macrophages. Further analysis is necessary to clarify a role, if any, for RICK in TLR signaling.

Pathogen-induced expression and secretion of multiple cytokines is mediated through several transcription factors activated by MAPK-related kinases in addition to NF- κ B. Nod1 has been reported to induce activation of c-Jun N-terminal kinases (JNKs) in response to bacteria (42), and macrophages deficient in Nod2 failed to activate extracellular signal-regulated kinase proteins (ERKs) upon muramyl dipeptide (MDP) stimulation (34). In addition, RICK has been implicated in p38/SAPK kinase activation through ERKs to induce phosphorylation of the transcription factor, Elk-1 (30, 43). These results indicate that, in addition to NF- κ B, Nod1 and Nod2 mediate the activation of JNK and p38 in response to microbial ligands, which are expected to participate in the transcriptional activation of proinflammatory genes. However, the molecular mechanism by which Nod1- and Nod2-mediated JNK and p38 activation is not well understood at this time.

Initially, several NOD-LRR proteins were identified by their structural homology to the apoptosis regulator, Apaf-1, suggesting that these proteins may regulate

apoptosis and/or caspase activation. Overexpression of Nod1, Nod2, Ipaf, or Cryopyrin has been shown to promote apoptosis and caspase activation (26, 28, 44–46). However, these NOD-LRR proteins also activate NF- κ B, which induces the expression of anti-apoptotic factors, including A1, c-IAPs, and c-FLIP (33, 47, 48). Therefore, it is possible that, under physiological conditions, apoptosis induced through NOD-LRR proteins might be inhibited by simultaneous induction of NF- κ B. Simultaneously, caspase and NF- κ B activation might be necessary to promote the production of proinflammatory molecules, such as IL-1 β and IL-18, which require caspase activation for processing and secretion.

The NOD-LRR proteins Ipaf, Cryopyrin, and NALP1 associate with ASC (TMS1/PYCARD), a molecule originally identified in the detergent-insoluble cytosolic fraction called the “speck,” in cells undergoing apoptosis (49). ASC is an adaptor protein composed of an amino-terminal PYD and a carboxyl-terminal CARD (49). With the exception of Ipaf, all of these NOD-LRR molecules contain an amino-terminal PYD that mediates the recruitment of ASC via PYD-PYD interactions. Cryopyrin and NALP1 have been found to promote, in the presence of ASC, the activation of caspase-1, interleukin-1 β -converting enzyme (44, 46, 50–52). A critical role for ASC in caspase-1 activation is also supported by analysis of mutant mice deficient in ASC. Macrophages lacking ASC are greatly impaired in their ability to activate caspase-1 and to secrete IL-1 β in response to multiple bacterial PAMPs (53).

Many of these ASC-associating NOD-LRR proteins may form large protein complexes to activate signaling. Biochemical experiments by Tschopp and coworkers (52) revealed that NALP1 forms a multiprotein complex, containing ASC, caspase-1, and caspase-5. This complex is dubbed “the inflammasome” and promotes activation of inflammatory caspases and the processing of prointerleukin-1 β (52). NALP1 is the only NOD-LRR family member with a carboxyl-terminal extension containing a CARD in addition to an amino-terminal PYD. In the NALP1 inflammasome, the PYD of NALP1 associates with the PYD of ASC, whereas the CARD recruits caspase-5. The CARD of ASC interacts with caspase-1, linking NALP1 to caspase-1. Immunodepletion experiments using a cell-free system revealed that ASC is required for caspase-1 and caspase-5 activation (52). In addition, there is also evidence that Cryopyrin can assemble in a large protein complex containing ASC, caspase-1, and CARDINAL in cell extracts of THP-1 monocytic cells (54). Like the NALP1 inflammasome, the Cryopyrin protein complex promotes caspase-1 activation. But, unlike NALP1, Cryopyrin is not involved in caspase-5 activation (54).

Ipaf is another NOD-LRR protein that has been implicated in caspase-1 activation (45, 46). In overexpression studies, the amino-terminal CARD of Ipaf has been shown to associate with the CARD of caspase-1 (45). This suggests a possible mechanism for caspase-1 recruitment via Ipaf, although it is also possible that Ipaf interacts with caspase-1 indirectly through ASC. Recent studies with Ipaf-deficient mice support a role for Ipaf in caspase-1 activation. Upon exposure to live *Salmonella typhimurium*, caspase-1 activation is impaired in macrophages

lacking ASC and Ipaf (53). These results suggest that multiple NOD-LRR family members are involved in the activation of inflammatory caspases, and several utilize the adaptor ASC for signaling.

ACTIVATION AND REGULATION OF NOD-LRR PROTEINS

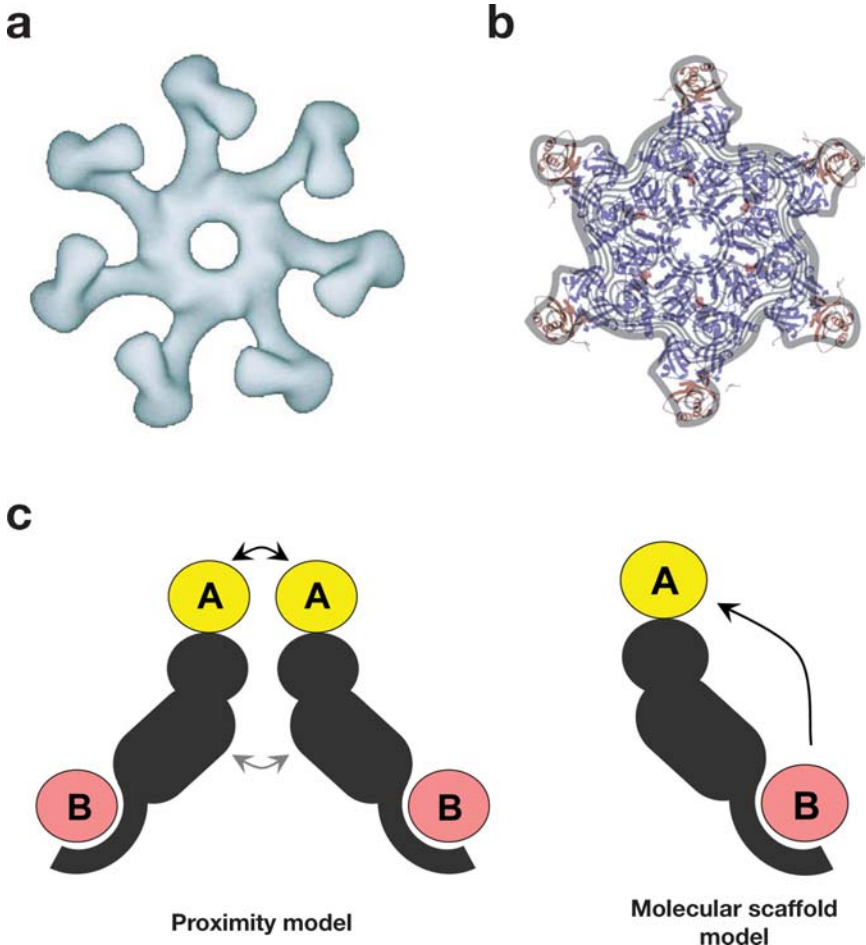
The structural similarities between Apaf-1 and NOD-LRR proteins suggest that these proteins may rely on similar mechanisms for activation and signaling. The activation of Apaf-1 has been extensively studied. In the cytosol, monomeric Apaf-1 is present in an inactive conformation, which prevents its oligomerization and association with caspase-9 (55–58). In response to many proapoptotic signals, cytochrome *c* is released from mitochondria, inducing Apaf-1 oligomerization and recruitment of caspase-9 (55, 56). In the presence of dATP/ATP, cytochrome *c* interacts with the WD-40 repeats, triggering the assembly of a large protein complex named the “apoptosome,” which contains Apaf-1 and caspase-9 (57, 59–61). Formation of the apoptosome causes the cleavage and activation of caspase-9, leading to apoptosis in the cell.

Amino acid sequence alignments revealed that the amino-terminal region spanning ~250 amino acids of the NOD domain in NOD-LRR proteins contains structural motifs found in a class of nucleoside triphosphatases (NTPases) referred to as the ATP-binding cassette (ABC) proteins. The ABC region in all Nod proteins includes catalytic residues with canonical binding site motifs for phosphate residues (Walker’s A box) and magnesium ions (Walker’s B box), as well as several motifs predicted to be involved in hydrolysis of nucleotides, ATP, deoxyATP (dATP) and/or GTP. Mutation of conserved residues in the Walker’s A and B boxes of Nod1 and Nod2 proteins abolished signaling, indicating that nucleotide hydrolysis is essential for protein activation (32). In the case of Apaf-1, however, dATP/ATP binding, but not hydrolysis, is essential for activation of caspase-9 (62). Therefore, it is possible that the conformational change between ATP/GTP- and ADP/GDP-binding states might be important for the function of NOD-LRR proteins, similar to the GTP- and GDP-bound states of small G proteins (63). The role of ATP hydrolysis per se in the regulation of NOD-LRR protein activity is presently unknown, but it might be important for the induction of conformational changes required for the return of the NOD-LRR protein to the original inactive state.

The ABC region in the NOD domain was originally referred to as the NACHT cassette in mammalian NOD-LRR proteins and as the NB-ARC domain in Apaf-1 and plant R proteins (64). The ABC region of NOD-LRR proteins is closely related to the oligomerization module found in the AAA+ family of ATPases (65). Cryo-electron microscopy revealed that oligomerized Apaf-1 forms a seven-spoked, wheel-like structure (66) (Figure 3a). This heptameric Apaf-1 structure closely resembles that of AAA+ proteins, which typically assemble into

ring-shaped oligomers with a six- or seven-fold symmetry (Figure 3*b*). In addition to Apaf-1, there is biochemical evidence for oligomeric assembly of NOD-LRR proteins (31, 67, 68), although the structure of these complexes is presently unsolved. These observations suggest conservation of molecular mechanisms among NOD-containing proteins and the AAA+ family of ATPases.

In the case of the apoptosome, oligomerization of Apaf-1 is thought to induce the proximity and dimerization of the associated monomeric zymogens of caspase-9, promoting their cross activation (55, 56, 69, 70) (Figure 3*c*). There is evidence that oligomerization of Nod1 induces the proximity of its interacting partner RICK (31), and that forced oligomerization of the effector domains of Nod1, Nod2, Cryopyrin, or Ipaf, as well as that of the downstream effector, ASC, induces signaling (28, 31, 44, 49, 71). Similarly, upon oligomerization of NALP1,



Cryopyrin, or Ipaf, caspase-1 molecules may be brought into close proximity, leading to self-activation. In another possible model, the different domains in a NOD-LRR protein may function as molecular scaffolds to recruit activators and effector molecules. Proximity of the activator to its target then promotes activation and thereby signaling. The latter model may be particularly relevant for proteins such as NALP1 and CIITA because they have been reported to recruit multiple signaling molecules (23, 52) (Figure 3c). Further studies are needed to understand the mechanisms of activation of NOD-LRR-mediated signaling.

DETECTION OF MICROBIAL COMPONENTS BY NOD-LRR PROTEINS

Plants contain a large number of NOD-LRR proteins to defend against a wide array of pathogens (5). Genetic evidence indicates that LRRs of plant NOD-LRR proteins act as pathogen recognition domains and interact with specific factors (elicitors) produced by the pathogen (72, 73). Unlike plants, there is evidence

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Figure 3 Structure of NOD-containing proteins and models of Nod protein activation. (a) Cryo-electron microscopy (cryo-EM) structure of oligomerized Apaf-1. The assembly of seven Apaf-1 molecules forms the apoptosome, which is a wheel-like particle with seven spokes connected to a central hub by an arm (*top view*). The WD40 repeats (cytochrome *c*-sensing domain) form the distal Y-shaped region of the spokes, whereas the NOD-like portion corresponds to most of the hub and the arm. This image is reproduced from Acehan et al. (66) with permission from Elsevier. (b) Model of the AAA+ protein p97 bound to its cofactor p47. Cryo-EM projection map (*top view*) of the full-length p97-p47 complex is shown. p97, which has been implicated in many different pathways ranging from membrane fusion to ubiquitin-dependent protein degradation, forms a hexameric ring to which six p47 molecules are bound to the peripheral protrusions via the N domain of p97. The centrally located ATPase domains of p97 provide an oligomerization surface for formation of the hexamer. The image is reproduced from Dreveny et al. (167) with permission from the publisher. (c) Two models of Nod protein activation include the induced proximity model (a) and the molecular scaffold model (b). For simplicity, side views are shown. In the induced proximity model, stimulatory ligands (factor B) are recognized through the ligand-recognition domain of Nods, which induces self-oligomerization and recruitment of downstream effector molecules (factor A) via the EBD. Oligomerization is mediated through the centrally located NOD domain (indicated by *gray arrow*), which induces proximity (indicated by *black arrow*) and activation of effector molecules, resulting in initiation of signaling. In the molecular scaffold model, the Nod protein recruits activator(s) (factor B) to activate (indicated by *arrow*) downstream signaling molecule(s) (factor A) bound to the EBD.

that Nod1 and Nod2 detect conserved structures shared by many pathogens. This observation may explain the vast difference between animals and plants in the number of NOD-LRR proteins.

Nod1 and Nod2 recognize peptidoglycan (PGN)-derived products through their carboxyl-terminal LRRs (33, 35, 39, 40, 74). PGN, a component of bacterial cell walls, is composed of glycan chains of repeated N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) units cross-linked to each other by short peptides (Figure 4). The cross-linking of two parallel glycan chains is mediated by the so-called stem peptides that can be further linked by bridging amino acids. Biochemical and functional analyses identified muramyl dipeptide (MDP) as the essential structure in bacterial PGN recognized by Nod2 (33, 74). MDP is present in practically all Gram-positive and Gram-negative bacteria (75). Thus, Nod2 acts as a general sensor of bacteria because MDP is part of the PGN structure that is conserved in common among nearly all bacteria. In contrast, Nod1 recognizes *meso*-diaminopimelic acid (*meso*-DAP)-containing PGN-related molecules (35, 40). DAP is an unusual amino acid that is unique to PGN structures from all Gram-negative bacteria and certain Gram-positive bacteria, including the genera *Listeria*

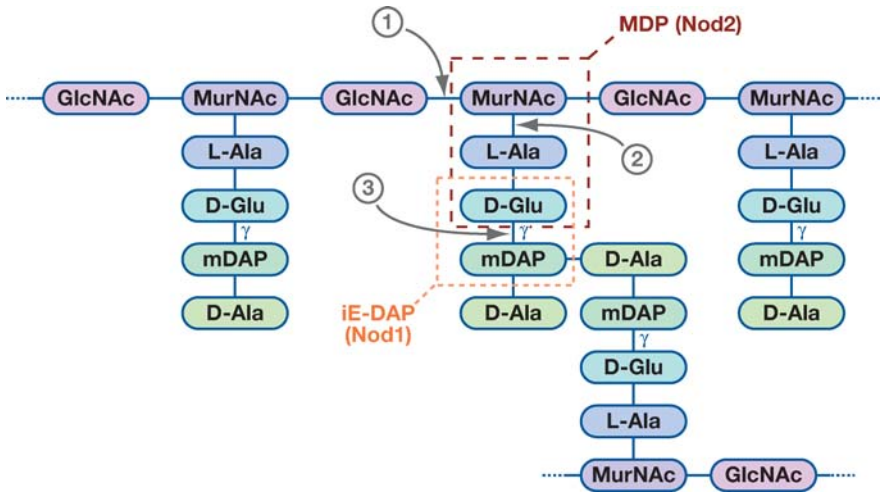


Figure 4 Generation of MDP and iE-DAP from bacterial peptidoglycan. In this simplified diagram of the PGN structure from *Escherichia coli*, parallel glycan strands composed of the alternating amino sugars, GlcNAc and MurNAc, are cross-linked to each other by stem peptides. Notice that *E. coli* PGN lacks bridging amino acids linking stem peptides, and cross-linking is made by a direct link between a *meso*-DAP residue and the D-Ala residue in position four from a peptide anchored on the parallel glycan strand. Minimal motifs required for Nod1 (orange box) and Nod2 (red box) recognition are shown. Numbers in circles 1 through 3 depict cleavage sites for muramidases (1), N-acetylmuramoyl-L-alanine amidases (2), and the p60 family of endopeptidases (3).

and *Bacillus* (75). Analysis of synthetic compounds revealed that the dipeptide γ -D-glutamyl-*meso*-DAP (iE-DAP) is sufficient to trigger Nod1 activation (35, 76). Macrophages isolated from mice deficient in Nod1 do not respond to iE-DAP but are responsive to TLR- and Nod2-stimulating PAMPs (35). Thus, Nod1 and Nod2 recognize PAMPs and act as PRMs.

The ligands for other NOD-LRR proteins remain to be identified. However, there are clues to what the potential ligands are for some of these proteins. Macrophages lacking Ipaf exhibit normal caspase-1 activation and IL-1 β production in response to various purified bacterial components, but they have impaired response to live *Salmonella* (53). Thus, it is possible that Ipaf is activated by a molecule expressed by *Salmonella* (and perhaps other bacteria) that is distinct from LPS, LTA, or PGN (53). The adaptor molecule ASC interacts with multiple NOD-LRR proteins (44, 45, 49, 77–79). Recent analysis of ASC-deficient mice revealed that macrophages from mutant mice exhibit impaired activation of caspase-1 and IL-1 β /IL-18 secretion in response to multiple TLR agonists (53). A possible interpretation of these results is that ASC acts as a common adaptor for multiple NOD-LRR proteins that are activated by these bacterial ligands. Alternatively, ASC could act downstream of multiple PRMs, including TLRs. Further examination is needed to elucidate the signaling pathway and microbial ligands for these NOD-LRR proteins.

GENERATION OF NOD1 AND NOD2 STIMULATORY MOLECULES

The core motif structures of Nod1- and Nod2-stimulatory molecules are found in PGN. However, insoluble, high-molecular-weight PGN is poorly stimulatory when compared to synthetic molecules containing the minimal structure required for Nod1 or Nod2 stimulation (33, 35). The length of the stem peptides that link the glycan chains in fragmented and intact PGN is not uniform and varies between two and five residues (80). There is evidence that the length of these peptides affects the Nod1- and Nod2-stimulatory activity (33, 35, 76). Generally, smaller PGN fragments possess more stimulatory activity than large molecules (33, 35), although the molecular basis that accounts for these differences in activity is presently not understood.

Bacterial and host enzymes are able to generate these PGN-related, small molecules with diverse mechanisms. In bacteria, small molecules containing iE-DAP or MDP structures are generated as intermediates of PGN biosynthesis (81–83). Bacteria also need to remodel PGN for cell division and growth, and in the process, generate small PGN fragments containing these motifs (80). In the host, lysozyme cleaves the glycan chain of PGN and generates small fragments called muropeptides, containing the iE-DAP and MDP structures. Lysozyme is present in body fluids, including blood and tears, as well as in the lysosomes of phagocytes that are involved in the degradation of PGN inside the cell (84, 85). In the acidic

environment of the lysosome, lysozyme can efficiently digest acetylated PGNs from several bacteria that are poorly cleaved in neutral fluids (86). In addition, lysozyme and other host factors induce bacterial lysis, leading to the release of small Nod1 and Nod2-stimulatory molecules from inside of the resulting bacterial bodies.

Both bacteria and host cells possess enzymes that hydrolyze the conserved MDP motif that is essential for Nod2 stimulation. These enzymes are N-acetylmuramoyl-L-alanine amidases that cleave the bond between the MurNAc sugar and the stem peptide. Host organisms also express high levels (about 0.1 mg/ml in human blood) of a liver-derived amidase, which has been found to be identical to the PGN-recognition protein L (87, 88). This amidase cleaves PGN in the blood and peritoneal fluid and generates iE-DAP-containing peptides (89, 90). The bacterial amidases, including *E. coli* AmiA and AmpD, are involved in the recycling of PGN, and their activity is expected to destroy the MDP structure, but not the iE-DAP motif responsible for Nod1-stimulatory activity (80). Bacteria also have DL-carboxypeptidase, which is expected to cleave the Nod1- and Nod2-stimulatory core structures (80). There is no evidence as yet for a host enzyme that can cleave iE-DAP, the essential Nod1-stimulatory motif. Indeed, almost all PGN fragments detected in blood and urine are derived from intact PGN or amidase-cleaved products (89, 90). However, engulfed PGN is metabolized in macrophages, and its sugars and alanine are incorporated into membrane fractions (91), suggesting the presence of an unidentified host enzyme that hydrolyzes the stem peptide and may lead to the release of the iE-DAP structure. In contrast, all bacteria are known to contain endopeptidases of the p60 family that are predicted to specifically cleave the iE-DAP structure (position 2 in Figure 4). p60 of *Listeria monocytogenes* is a critical virulence factor that might help avoid innate immune recognition (92). Thus, through degradation of iE-DAP, p60 and related bacterial endopeptidases might play a role in evading Nod1-mediated host recognition.

The Nod1-stimulatory activity recovered from bacteria is highly stable, when compared to MDP and most TLR ligands. It is extremely resistant to heat, acidic, alkaline, and organic extraction treatments (35). Notably, these harsh treatments and physical disruption of bacteria degrade PGN and release iE-DAP-containing small fragments with Nod1-stimulatory activity (35, 40). The high stability of the Nod1 ligand suggests that the activity may be present in foodstuff prepared by bacterial fermentation and foods contaminated with bacteria, even after sterilization of the contaminated food. In addition, the high stability of the core iE-DAP structure explains why many commercial preparations of PGN, LPS, and possibly of other bacterial components contain Nod1-stimulatory molecules (33, 35, 39–41). These observations impact on the interpretation of previous work in which the immunostimulatory function of impure PGN and LPS preparations were studied, in that the results may reflect, at least in part, additive or synergistic effects of TLR- and Nod1-stimulatory molecules. Unfortunately, a convenient assay, like the Limulus test for LPS, has not been developed for Nod1-stimulatory molecules.

RECOGNITION OF MICROBIAL LIGANDS BY NOD-LRR PROTEINS

The mechanism by which cytosolic Nod1 and Nod2 mediate the recognition of MDP and iE-DAP-containing molecules is poorly understood. The observations that addition of MDP and iE-DAP in culture medium can elicit Nod2- and Nod1-dependent signaling, respectively (33, 35, 40, 74, 76), and that PGN is metabolized inside macrophages (91) suggest the presence of an active transport system for the delivery of PGN-related small molecules into the cytosol. There is evidence that MDP can be transported by pepT1, a protein involved in transport of nutrient di- or tri-peptides across the plasma membrane of intestinal epithelial cells (93). However, further studies are required for the identification of transport proteins involved in the uptake or entry of natural Nod1- and Nod2-stimulatory molecules into epithelial and immune cells.

The LRRs of plant and animal NOD-LRR proteins are involved in pathogen recognition and can be considered the ligand-sensing domain. LRRs are composed of a repeated 20- to 29-residue long motif with a unique α/β -folding present in a large number of proteins with diverse functions (94). Crystal structures of several LRR-containing proteins revealed that the LRRs are arranged in a horseshoe structure with a curved parallel β -sheet lining the concave surface (94). In plant Nod proteins, the LRR domain is the most variable region, presumably because it is under diversifying selection driven by pathogens (5, 72, 73). Mutational analyses of Nod1 and Nod2 proteins have revealed critical amino acid residues, required for recognition of their microbial ligands that are located in the LRRs (32). Within the carboxyl-terminal LRRs of Nod2, variable residues within the predicted β -strands of the LRRs were found to be essential for MDP recognition (32). Similarly, analyses of chimeric genes have implicated the same nonconserved residues forming the β -strand/turn (xxLxLxxx) LRR motif in the recognition of pathogen elicitors by plant NOD-LRR proteins (95).

The variable residues located on the β -sheet surface are known to mediate the interaction of other LRR proteins with their binding partners (94). However, in the case of Nod1 and Nod2, it is not known if iE-DAP or MDP bind directly or require cofactors for binding and activation. Purified Nod1 protein was originally found to associate with radiolabelled LPS fraction from *E. coli* (39), but the molecule responsible for the binding activity in the preparation has not been identified. There is also evidence that the cytosol of certain cells contains MDP-binding activity, and the molecular weights of major MDP-binding proteins have been reported to be in the 40–45 kDa range (96). Thus, Nod2 (~115 kDa) might bind MDP that is already in complex with this cellular factor. Clearly, further work is necessary to elucidate the mechanism by which NOD-LRR proteins recognize their microbial ligands.

Ligand recognition through the LRRs is expected to relay signals leading to the activation of the NOD domain. Complementation analysis of mutants has demonstrated functional interactions between amino acid residues located in the

proximal LRRs (737 to 854 in Nod2 protein) and the distal region of the NOD domain (584-664 to 736 in Nod2 protein) (32). This most carboxyl-terminal part of the NOD is referred in this review as the “switching region” in that it is suggested to play a role in turning an inactive Nod protein to its active state (32). Consistent with the presence of a negative regulatory element in the switching region of NOD-LRR proteins is the finding that point mutations in several amino acids located in this region or truncation of this region lead to activation of mammalian and plant NOD-LRR proteins in the absence of any source of stimulatory ligands (32, 97–99). Missense mutations in this carboxyl-terminal region of the NOD of Cryopyrin also result in constitutive activation because such mutations are causing inflammatory disease (see below), underscoring the physiological relevance of the switching region. Analysis of truncation mutants has revealed a region encompassing the distal segment of the switching region and the most proximal LRRs that mediates inhibition of Nod1 and Nod2 activity (32). A similar inhibitory region has been identified in the corresponding portion of plant NOD-LRR proteins (97–99). These observations suggest a model in which the negative regulatory function might be mediated by intramolecular interactions between the proximal part of the LRRs and the distal part of the NOD domain, and upon exposure to microbial ligands, the resulting conformational change may reverse the inhibition, resulting in NOD activation and signaling.

ROLE OF NOD1 AND NOD2 IN IMMUNITY

The function of Nod1 and Nod2 in innate and adaptive immunity is poorly understood. However, multiple studies performed over the past 20 years with purified and synthetic PGN-derived molecules have provided insight into the biological functions of Nod1 and Nod2. Administration of MDP and iE-DAP derivatives induces broad activity against multiple pathogens, including Gram-negative and Gram-positive bacteria, fungi, viruses, protozoa, and tumor growth in animal models (100). Antibody neutralization studies suggested that this wide range of microbicidal activity could be explained by the induction of the key proinflammatory cytokines, TNF and IL-1 (101–105). PGN-derived compounds have also been shown to induce production of reactive oxygen species and pathogen-killing activity in macrophages (106, 107). iE-DAP derivatives also have been demonstrated to induce the cytotoxicity of IL-2-activated lymphocytes (108, 109). Recent studies have indicated that MDP induces NF- κ B activation and that practically all MDP-inducible genes require functional Nod2 (36). MDP is known to induce secretion of multiple mediators, including proinflammatory cytokines (TNF α , IL-1 α , IL-1 β), chemokines (e.g., IL-8, CCL3, CCL4), hematopoietic growth/survival factors (IL-6, G-CSF, GM-CSF), and also synthesis of nitric oxide synthase, all of which are critical for the innate immune response against pathogens (36, 101–105, 110). MDP also induces expression of adhesive molecules (ICAM1, CD44, TNFAIP6), which are important for recruitment of inflammatory cells to infected

sites (110). Thus, both MDP and iE-DAP stimulate several immune responses that are important for pathogen clearance.

MDP is known to be an adjuvant that stimulates the generation of antigen-specific T- and B-cell responses, delayed-type hypersensitivity, and antibody production (100). Similarly, iE-DAP derivatives have been reported to confer adjuvant activity in animal models (100). This adjuvant activity may be explained, at least in part, by the fact that MDP induces expression of costimulatory molecules (CD40, CD80, CD86) in monocytes and dendritic cells, which mediate differentiation of naive T cells into effector T cells (110–112). Use of stereoisomeric MDP analogs has indicated a good correlation between Nod2 recognition and adjuvant activity (33, 74). These results suggest that Nod2, which is expressed in monocytes and dendritic cells (28, 113), plays a role in coupling the innate and the adaptive immune responses in a similar manner to that reported for TLRs. However, MDP induces secretion of IL-12 and interferon- γ (IFN- γ) only poorly. Both IL-12 and IFN- γ are important for the induction of adjuvant activity by TLR ligands.

MDP synergizes with LPS in the production of cytokines (114–116), suggesting that TLRs and NOD-LRR proteins cooperate in immune responses against bacteria. The molecular basis for the synergistic effect is unclear, but it may be caused by cross induction of the signaling components needed by the NOD-LRR- and TLR-mediated pathways. For example, LPS upregulates the expression of Nod2, RICK, Cryopyrin, and ASC, whereas MDP upregulates MyD88, a critical molecule in TLR signaling (113, 115). Curiously, the strength of the immune response to MDP and iE-DAP derivatives varies greatly depending on the animal species and genetic background of the animal strain. For example, mice are much less sensitive than humans, guinea pig, or rats to these PGN-derivatives, and C57BL/6 and C57BL/10Sn mice are less sensitive than ICR or BALB/C mouse strains (117, 118). The differential sensitivity of mice to MDP and iE-DAP does not relate to genetic Nod1 and Nod2 polymorphisms observed in different mouse strains (119). In animals with low sensitivity, the immune response to MDP and iE-DAP derivatives is poor and is detected better through synergism with TLR ligands. Therefore, Nod1 and Nod2 stimulation may appear to act primarily as a regulator of TLR signaling in low responding animals. The synergism between Nod1/Nod2 and TLR signaling might be particularly important in vivo when the amount of TLR ligands may be limiting.

Pathogenic bacteria have developed a variety of strategies to avoid host recognition and immune responses. Gram-negative bacteria possess an outer membrane, which covers and physically isolates PGN and related small molecules from recognition by host factors. Some bacteria contain capsular structures to avoid direct contact of immunostimulatory molecules on the bacterial surface with host recognition factors. PGN fragments from several Gram-positive bacteria including *Bacillus licheniformis* and *Bacillus subtilis* are modified via amidation at D-Glu and *meso*-DAP residues, respectively, and possess reduced Nod1-stimulatory activity when compared to their amidation-free counterparts (35, 40, 83). These bacterial and host modifications may have evolved to escape host response initiated

by Nod1-mediated recognition. The latter mechanism is reminiscent of the acyl modification of *Salmonella* LPS that helps to avoid host recognition through TLR4 and other antibacterial molecules (120). Amidated amino acids are also involved in peptide bridging to other strands of PGN, and both the structure and frequency of the bridging moieties vary highly among bacterial species. Further studies using bacteria with different PGN structures are required to understand the bacteria/host interaction though PGN-related molecules and Nod1 and Nod2.

NOD-LRR PROTEINS AND DISEASE

Genetic variation in three human NOD-LRR proteins has been implicated in the development of disease (14). Two types of disease-causing variants, loss-of-function and constitutively active mutations, have been observed. In some cases, these mutations involve homologous residues in different NOD-LRR proteins, suggesting a common molecular mechanism of disease. Loss-of-function mutations in CIITA cause type II bare lymphocyte syndrome (BLS), a hereditary disorder characterized by MHC-II deficiency (21, 23). These mutations impair the ability of CIITA to act as a transcriptional activator and thereby reduce MHC-II expression (23, 121). Because MHC-II molecules play a critical role in antigen presentation to T lymphocytes and therefore host defense against pathogens, patients with BLS suffer from severe primary immunodeficiency and succumb to repeated viral and/or bacterial infections in early childhood.

Mutations in the *NOD2* gene have been implicated in two inflammatory diseases. Three major *NOD2* mutations, R702W, G908R, and L1007fsinsC, as well as multiple rare variants, have been found to be associated with susceptibility to Crohn's disease (CD), a chronic, relapsing inflammatory disease of the bowel characterized by granulomatous inflammation, primarily localized to the terminal ileum. In addition, the major *NOD2* mutations found in CD have been reported to be associated with several different immune diseases, including development of sepsis in infants (122), allergic disease (123), psoriatic arthritis (124), radiological sacroiliitis (125), and graft-versus-host disease (126). These CD-associated Nod2 variants are deficient in their ability to sense PGN and/or synthetic MDP (33, 74). A role for defective Nod2 signaling in CD is also supported by analysis of peripheral blood mononuclear cells (PBMC) from individuals homozygous and heterozygous for the common Nod2 mutations. CD patients or healthy individuals homozygous for the main L1007fsinsC *NOD2* mutation are impaired in their NF- κ B response to MDP but not to LPS (33). Furthermore, cultured macrophages derived from the PBMC of CD patients homozygous for R702W, G908R or L1007fsinsC exhibit a profound defect in the MDP-dependent induction of proinflammatory cytokine and chemokine genes, as determined by microarray gene analysis (36). In contrast, individuals heterozygous for *NOD2* mutations exhibit a normal response to MDP (33). Consistent with these functional studies, homozygosity and compound heterozygosity increase up to ~40-fold the genotype relative risk for CD compared to

simple heterozygosity (~2–4-fold) (127–129). In Western populations, over 50% of CD patients possess one of the major *NOD2* mutations and ~5% to 20% of the patients are homozygous or compound heterozygous for the same mutations, indicating that the *NOD2* locus plays an important role in disease susceptibility. In addition to *NOD2*, several loci have been linked to CD, two of which, *IBD4* and *IBD6*, are associated with disease independently of *NOD2* alleles (130, 131). Notably, *NOD2* mutations are associated with early-onset and ileal involvement of disease but much less with disease in which the ileum is spared (132–137), suggesting that CD can be subclassified according to *NOD2* genotype.

The precise mechanism by which loss-of-function Nod2 mutations increase the susceptibility to CD is not understood. Certain individuals homozygous for the main *NOD2* mutations are healthy, suggesting that in addition to Nod2, other genetic and/or environmental factors contribute to the development of CD (33, 138). There is evidence that epistatic effects of *IBD5* (*Octn-1* alleles), *IBD7*, *CD14*, and *DLG5* alleles with *NOD2* alleles play a role, but are not sufficient for CD development (139–146). The observation that the response to bacterial components is impaired in CD-associated *NOD2* variants suggests that a deficit in sensing certain bacteria by Nod2 may trigger secondary intestinal inflammation, perhaps in response to uncleared bacteria or bacterial products in the lamina propria. In this model, Nod2 actually plays a protective role in host defense, and CD is triggered by innate immunodeficiency caused by defective bacterial recognition. In the intestine, epithelial cells lining the lumen and resident macrophages in the lamina propria are largely insensitive to bacterial components. However, Nod2 is primarily expressed in monocytes and a subset of macrophages in the lamina propria, which are capable of sensing bacteria. Paneth cells, located in the crypts of the ileal mucosa, are specialized epithelial cells that respond to bacterial products by secreting antibacterial peptides (147). Intriguingly, Nod2 is expressed in Paneth cells and might regulate the antibacterial function of Paneth cells, an activity that may be impaired in CD patients (148, 149). Indeed, Nod2 regulates the expression of certain α -defensins, a family of antimicrobial peptides produced by Paneth cells in humans and mice (150, 151). Furthermore, Nod2-deficient mice showed impaired protection against oral, but not intravenous or intraperitoneal, administration of *L. monocytogenes*, suggesting a specific role of Nod2 in intestinal immunity (151). Thus, impaired production of α -defensins in Paneth cells due to Nod2 mutations might be the link between Nod2 and susceptibility to CD. Another, but less likely, possibility to explain the role of Nod2 in this inflammatory disease is that Nod2 plays a negative regulatory role in the immune response to bacteria, and Nod2 deficiency could lead to inappropriate activation of T cells in intestinal tissues. Supporting this model, macrophages from Nod2-deficient mice exhibit increased cytokine response, including increased IL-12 secretion in response to TLR2 ligands (37). In contrast, however, in CD patients homozygous for the L1007fsinsC mutation, the response to TLR2 stimulation is impaired, including IL-10 production (38). Moreover, another study showed normal TLR2 signaling in Nod2-deficient mouse macrophages (151). Clearly, further work is

needed to understand the mechanism by which loss-of-function *NOD2* mutations promote CD.

In addition to CD, several missense mutations of Nod2, R334Q, R334W, and L469F have been associated with the development of Blau syndrome (BS), a monogenic, dominantly inherited disease characterized by early-onset granulomatous arthritis, uveitis, and skin rashes (152). The disease-causing mutations involve residues located in the NOD domain of Nod2 and exhibit constitutive NF- κ B activity compared to wild-type Nod2 (32, 41). Although both CD and BS are characterized by the presence of granulomatous inflammation, they are clearly different diseases with distinct clinical presentation and mode of inheritance (153). Unlike CD, there is no evidence that bacteria play any role in the pathogenesis of BS. Thus, inflammatory disease associated with BS is likely induced by the constitutive activation of Nod2.

Three autosomal-dominant diseases, familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), and chronic infantile neurological cutaneous and articular (CINCA) syndrome, are associated with missense mutations in the *CIAS1* gene, which encodes Cryopyrin (154, 155). The Cryopyrin-associated diseases represent closely related autoinflammatory syndromes, characterized by recurrent episodes of fever, skin rashes, and tissue inflammation (155–158). Disease-causing missense mutations involve residues extending throughout the entire NOD domain of Cryopyrin. Several mutations, including R260W and D303N, are located in the ABC region of the NOD domain, potentially affecting the active conformation of Cryopyrin. R260W, another *CIAS1* mutation associated with FCAS and MWS, corresponds to the R334W *NOD2* gain-of-function mutation found in BS (153). Additional mutations, including E637G, are found in an uncharacterized region located between the ABC region and switching regions of Cryopyrin. Notably, some disease-causing mutations correspond to identical or closely located residues in the switching region of Nod2, wherein mutation results in constitutive activation (32). Functional studies have revealed that the disease-associated *CIAS1* mutants, R260W, D303N, and E637G, exhibit a gain-of-function phenotype with enhanced ASC recruitment, NF- κ B activation, and IL-1 β secretion when compared to the wild-type protein (79). Consistent with these studies, it was found that unstimulated blood cells from patients with MWS and CINCA secreted constitutively elevated levels of proinflammatory cytokines, including IL-1 β (54, 158). An important role for dysregulated IL-1 β in disease pathogenesis is supported by clinical improvement after treatment of MWS patients with IL-1 receptor antagonist (159). The molecular mechanism that confers constitutive activation to disease-associated *CIAS1* and *NOD2* mutations is presently unclear. The residues mutated in these autoinflammatory diseases are predicted to play an important role in the regulation of the NOD domain and Cryopyrin/Nod2 activity, and the disease-causing mutations may stabilize the active conformation of the NOD-LRR proteins.

Pyrin (Marenstrin), the product of *Familial Mediterranean fever* (FMF) gene (*MEFV*), has been recently implicated in the regulation of NOD-LRR protein

signaling and inflammatory disease. FMF is an autosomal recessive disease, characterized by recurrent episodes of fever and localized inflammation (160, 161). Pypin is composed of an amino-terminal PYD, a B-type zinc-finger box, a coiled coil, a PRY domain, and a Spla and Ryanodine receptor (SPRY) domain (160, 161). The PYD of Pypin physically interacts with the PYD of ASC (44, 49, 162) and, when Pypin is overexpressed, specifically inhibits Cryopyrin- and Ipaf-mediated signaling (44, 49). Mice with a FMF-associated mutation of Pypin exhibit heightened sensitivity to LPS and produce increased amounts of activated caspase-1 and mature IL-1 β upon LPS stimulation (163). Thus, Pypin may act as a negative regulator of Cryopyrin and/or Ipaf signaling and loss-of-function mutations in patients with homozygous *Pypin* mutations may lead to enhanced inflammation in FMF. The mechanism by which Pypin negatively regulates Cryopyrin and/or Ipaf signaling is presently unclear. Pypin may act, at least in part, by disrupting the Cryopyrin-ASC interaction (44) and thereby inhibit ASC-dependent signaling. Remarkably, the major human *Pypin* mutations are located in the SPRY domain, which is missing in Pypin of rodents (160, 161, 163). The latter observation is puzzling and prompts additional studies to understand how these mutations impact ASC and related signaling pathways.

In addition to the human disease-associated NOD-LRR protein mutations, genetic variation in one mouse NOD-LRR protein has been associated with immune regulation defects. NAIP was originally described as an inhibitor of neuronal apoptosis on the basis of its putative involvement in spinal muscular atrophy, a disease characterized by neuronal degeneration (22). However, the disease appears to be caused by mutations in *SMN1*, a neighboring gene at the chromosomal region 5q13, and NAIPs are expressed primarily in macrophages and other immune cells but not in neuronal tissue (Table 1). Mice have seven *NAIP* paralogues, *naip1* to *naip7*, located in the same region of chromosome 13 (164, 165). Mouse NAIP proteins share extensive amino acid identity, and there is evidence that genetic variation in the *naip5* gene regulates permissiveness for intracellular replication of *Legionella pneumophila* in mouse macrophages (15, 16). The mechanism by which Naip5 regulates intracellular growth of *L. pneumophila* remains elusive. There are numerous missense polymorphisms in the coding region *naip5* gene that could account for permissiveness differences among mouse strains (16). Further work is necessary to understand the critical residues involved and the mechanism by which Naip5 regulates intracellular growth of *L. pneumophila* in macrophages.

PERSPECTIVES

The identification and initial characterization of cytosolic NOD-LRR proteins in mammals have widened our view on the host recognition system involved in microbial detection and inflammatory defense mechanisms. The involvement of NOD-LRR proteins in the pathogenesis of several genetic diseases indicates that these proteins play a pivotal role in the regulation of immune and inflammatory

responses. The finding that Nod1 and Nod2 recognize conserved PGN-derived molecules indicates that at least some of the NOD-LRR proteins function as PRMs for the recognition of microbes. Several NOD-LRR proteins appear to be involved in the regulation of NF- κ B and caspase activation. Further studies including functional analyses of gene knockout mice are necessary to understand their biological roles in immune responses. Many issues still remain, including the identification of microbial and/or endogenous ligands for many NOD-LRR proteins, the mechanism involved in ligand recognition through the LRRs, and the characterization of activation and downstream events mediated by NOD-LRR proteins. In addition, a better understanding is needed of the mechanism by which Nod2 and Cryopyrin mutations correlate with susceptibility to inflammatory disease. Resolution of these issues could provide important clues for the development of more rational therapies for NOD-LRR-associated diseases.

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