Nod1 and Nod2 in innate immunity and human inflammatory disorders

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Abstract
Nod (nucleotide-binding oligomerization domain) 1 and Nod2 are intracellular PRMs (pattern-recognition molecules) of the NLR (Nod-like receptor) family. These proteins are implicated in the detection of bacterial peptidoglycan and regulate pro-inflammatory pathways in response to bacteria by inducing signalling pathways such as NF-κB (nuclear factor κB) and MAPKs (mitogen-activated protein kinases). The Nod proteins act independently of the TLR (Toll-like receptor) cascade, but potently synergize with the latter to trigger innate immune responses to microbes. Most importantly, mutations in Nod2 have been shown to confer susceptibility to several chronic inflammatory disorders, including Crohn’s disease, Blau syndrome and early-onset sarcoidosis, underscoring the role of Nod2 in inflammatory homeostasis. This review summarizes the most recent findings in the field of Nod1 and Nod2 research.

Introduction
Sensing and defeating microbial infections is essential for the survival of metazoan species. All living organisms are constantly exposed to microbes that are, most of the time, neutral or beneficial to the host, but sometimes cause infectious diseases. To control the colonizing microflora and combat the pathogens effectively, multicellular organisms have developed a variety of defence mechanisms. These include physical (skin, mucosal lining), mechanical (tight junctions) and biochemical (antimicrobial enzymes) barriers, as well as, in vertebrates, the active participation of the adaptive immune system.

The innate immune system, as the first line of defence against microbial pathogens, constantly monitors the host’s environment. A critical step in the immune response is the identification of an invading organism as foreign. This recognition step involves interactions between microbial structural motifs and host receptors. Recent studies have shown that the innate immune system has a greater degree of specificity than was previously thought and that it is highly developed in its ability to discriminate between the self and foreign pathogens. This discrimination relies on a family of evolutionary conserved receptors called PRMs (pattern-recognition molecules) that recognize a limited, but highly conserved, set of molecular structures that are produced by micro-organisms and are absent from the host cell. A first family of PRMs, the TLRs (Toll-like receptors), is composed of membrane-anchored proteins that detect a variety of extracellular (or intravesicular) MAMPs (microbial-associated molecular patterns) or DAMPs (danger-associated molecular patterns) [1]. In addition, recent studies in mammalian systems have indicated the presence of intracellular PRMs called NLRs [Nod (nucleotide-binding oligomerization domain)-like receptors] that, as for TLRs, sense and respond to MAMPs or DAMPs in the cytosol [2,3].

NLRs
NLRs are a growing family of regulatory proteins that have a conserved tripartite domain structure: the C-terminal LRR (leucine-rich repeat) domain that is likely to recognize ligands (directly or indirectly), the NACHT domain present in NAIP (neuronal apoptosis inhibitory protein), CIITA (MHC class II transcription activator), HET-E (incompatibility locus protein from Podospora anserina) and TP1 (telomerase-associated protein) that mediates oligomerization, and the N-terminal effector domain that generates downstream signalling (Figure 1). LRRs consist of motifs with a length of 20–29 amino acids. The number of repeats varies among proteins, and the LRRs of NLRs are homologous with those of plant disease-resistance proteins (R-proteins) and TLRs, which raises the possibility that they also trigger immune responses upon recognition of specific structures. In mammals, the NLR family is composed of 22 members (see Figure 1) that can be grouped on the basis of the presence of the NACHT and LRR domains. Most of the diversity in this family comes from the N-terminal region. A first N-terminal

Key words: inflammation, innate immunity, microbial infection, Nod-like receptor (NLR), nuclear factor κB (NF-κB), pattern-recognition molecule (PRM).

Abbreviations used: CARO, caspase recruitment domain; CFA, complete Freund’s adjuvant; CHBE, H. pylori translocator; DAMP, danger-associated molecular pattern; dsDNA, DNA; HSP90, heat-shock protein 90; IL, interleukin; iNOS, inducible NO synthase; LRR, leucine-rich repeat; MAMP, microbial-associated molecular pattern; NAIP, neuronal apoptosis inhibitory protein; NACHT, LRR/pyrin domain-containing protein; NF-κB, nuclear factor κB; Nod, nucleotide-binding oligomerization domain; NLR, Nod-like receptor; PRR, pattern-recognition molecule; PVD, pyrin domain; RIP2, receptor-interacting protein 2; SGT1, suppressor of G2 allele of Skp1; TLR, Toll-like receptor; TNF, tumour necrosis factor.

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domain, named CARD (caspase recruitment domain) is found in Nod1, Nod2 (Nod2 has two N-terminal CARD domains) and IPAF (ICE [IL (interleukin)-1β-converting enzyme] protease-activating factor). An N-terminal PYD (pyrin domain) is found in the largest subgroup of NLR proteins, the NALPs (NACHT/LRR/PYD-containing proteins). CIITA and NAIP (named Naip5 in mice) display an N-terminal AD (activation domain) and BIR (baculovirus inhibitor of apoptosis protein repeat) domains respectively. Finally, three NLR proteins, Nod3, Nod9 and Nod27, display N-terminal sequences with no homology with known domains. It is well documented that NLRs mediate detection of PAMPs (pathogen-associated molecular patterns) or DAMPs via their LRR region (see also below). Interestingly, a phylogenetic analysis of the LRR domain of all human NLRs shows that, whereas NALPs form a homogeneous group, Nod3, Nod9 and Nod27 seem to be closer to Nod1 and Nod2 in terms of their LRR architecture (S.E. Girardin, unpublished work). This may suggest that these uncharacterized members of the NLR family could function in a similar way to Nod1 and Nod2.

NLRs are believed to remain in an inactive form until the detection of a ligand by their LRR domain, resulting in conformational changes that unlock the N-terminal domains for downstream signalling. The activated NLR may then serve as a molecular platform for protein complexes such as inflammasomes (for NALPs) or nodosomes (for Nods) by promoting the activation of downstream effector molecules through self-association and induced proximity of binding partners. The activation regulates intracellular signalling pathways [such as NF-κB (nuclear factor κB), caspase 1 inflammasome and MAPKs (mitogen-activated protein kinases)], which leads to altered gene expression of proteins involved in inflammatory responses, cell fate or antimicrobial activity [2,3]. Furthermore, through adaptors and caspase 1, they also regulate the cleavage of pro-inflammatory cytokines such as pro-IL-1β or pro-IL-18.

A number of human diseases, such as cancers and autoinflammatory diseases, are linked to polymorphisms, mutations or promoter hypermethylation of NLRs, which strengthens the idea that these molecules are important in inflammation and immunity. In particular, genetic analyses have demonstrated that Nod2/CARD15 mutations were linked to susceptibility to Crohn’s disease, a chronic inflammatory disorder affecting the intestinal tract (see below). In addition, several rare inflammatory disorders have been linked to mutations in Nalp3, including Muckle–Wells syndrome, CINCA (chronic infantile neurologic cutaneous and articular disease) and FCAS (familial cold auto-inflammatory syndrome) (also known as familial cold urticaria).

Several recent review articles have extensively described the newest concepts in NLR biology [2–4]. For the purpose of the present review, we will now focus our attention on Nod1 and Nod2, two of the best characterized members of the NLR family of intracellular PRMs.

**Nod1 and Nod2 as sensors of bacterial peptidoglycan**

Nod1 and Nod2 detect distinct sub-structures from bacterial peptidoglycan. Whereas Nod2 detects MDP (muramyl dipeptide; MurNAc-L-Ala-D-isoGln), the largest peptidoglycan motif common to Gram-negative and Gram-positive bacteria [5–7], Nod1 senses meso-DAP (meso-diaminopimelic acid)-containing peptidoglycan [8,9], which is more commonly found in Gram-negative bacteria. The optimal peptidoglycan motifs detected by human and murine Nod1 are TriDAP (L-Ala-D-Glu-γ-DAP-D-Ala)-containing structures respectively, and the minimal activating structure is the dipeptide iE-DAP (γ-D-Glu-meso-DAP) [6,8].

The mode of presentation of peptidoglycan to Nod1 and Nod2 remains unclear. There is no evidence to date that these NLRs bind directly to peptidoglycan, and it is possible that as yet uncharacterized adaptor proteins may bridge these two entities. The question of how peptidoglycan could reach the cytosol is still an enigma, except in the case of the infection with an invasive bacterium that escapes into the host cytosol (such as *Shigella* or *Listeria*) and thus serves as a vehicle to internalize peptidoglycan (Figure 2). hPepT1 (human peptidase transporter 1), a transmembrane transporter of di- or tri-peptides, has been shown to internalize MDP [10], but is unable to transport Nod1-activating agonists or Nod2-stimulating muramyl peptides larger than MDP [11], suggesting that alternative mechanisms of transport of peptidoglycan must exist in cells (Figure 2).
Activation of Nod1 and Nod2 signalling pathways

The ligands of Nod1 and Nod2, DAP-type peptidoglycan (DAP-PG) and MDP respectively can be presented intracellularly to Nod proteins by four principal means: 1, following infection with an invasive bacterial pathogen; 2, through secretion system-mediated translocation of muropeptides; 3, by host-cell-mediated internalization, via mechanisms that have not yet been elucidated; 4, via hPepT1, in the case of MDP. Following detection of their respective peptidoglycan ligands, Nod1 and Nod2 trigger downstream signalling pathways via RIP2. Several proteins have been shown to interact with Nod1, Nod2 or RIP2 and modulate signalling, either positively or negatively, as indicated. GRIM-19, gene associated with retinoid-interferon-induced mortality 19.

Signalling pathways triggered by Nod1 and Nod2

Activation of Nod1- and Nod2-dependent pathways results in the induction of the NF-κB pathway. In addition, a direct interaction between Nod2 and NIK (NF-κB-inducing kinase) has been reported to trigger the p100/p52-dependent induction of the non-canonical NF-κB pathway in murine primary macrophages [12]. Nod1- and Nod2-dependent pathways also stimulate JNK (c-Jun N-terminal kinase) [13,14] and p38 MAPK [12] (Figure 2).

Despite more than three decades of use in various biological systems, the specific Nod1 and Nod2 peptidoglycan agonists have not been shown to induce apoptosis pathways, with the exception of one study [15]. It is plausible that Nod pathways could turn on apoptosis pathways (such as caspase induction), but that the final outcome of cell death would be generally masked by the overriding anti-apoptotic power of Nod-mediated activation of NF-κB pathways. Accordingly, in studies where activation of survival pathways was impaired by cycloheximide treatment, Nod agonists could efficiently trigger caspase-dependent apoptosis, implicating caspase 8 specifically [16].

The Nod-dependent signalling pathway is far from being fully characterized. The current model shows that the oligomerization of Nod proteins following peptidoglycan sensing results in the recruitment of RIP2 (receptor-interacting protein 2), also known as RICK [Rip-like interacting CLARP (caspase-like apoptosis-regulatory protein) kinase], which in turn interacts with the IKK [IκB (inhibitor of NF-κB) kinase] complex, the converging knot of common NF-κB-activating pathways, including TLR-, IL-1- and TNF (tumour necrosis factor)-mediated signalling cascades. A number of proteins have been shown to interact with Nod1, Nod2 or RIP2 and/or to modulate Nod-dependent signalling (Figure 2). In overexpression studies, CARD6 has been shown to bind RIP2, but affected Nod-dependent NF-κB activation only modestly [17]. Similarly, overexpressed TAK1 [TGF (transforming growth factor)–β-activated kinase 1] and Nod2 have been shown to interact [18]. Two studies have found, using unbiased screens for identifying new Nod partners, that overexpressed Nod1 and Nod2 interact with SGT1 (suppressor of G2 allele of Skp1), a co-chaperone of HSP90 (heat-shock protein 90) [14,19]. Since overexpressed Nod proteins tend to aggregate, it is possible that the recruitment of the HSP90 complex is a rather artificial result of folding stress in transfected cells. It will be of interest to analyse the interaction of SGT1 with endogenous Nod proteins. Two other studies have reported the interaction between Nod2 and Erbin, again relying on unbiased screens [20,21]. Indeed, the study of McDonald et al. [20] used tandem affinity purification, and the one from Kufer et al. [21] used the yeast two-hybrid technique. Interestingly, both reports identified Erbin as a negative regulator of Nod2-dependent signalling. Finally, using a yeast two-hybrid screen, Barnich et al. [22] identified GRIM-19 (gene associated with retinoid-interferon-induced mortality 19) as a Nod2-interacting protein that is important for Nod2-mediated responses [22].

Nod1 and Nod2 in human diseases

Several NLRs have been linked genetically to inflammatory disorders. One of the first associations reported has been between nod2 and Crohn’s disease [23,24], a form of IBD (inflammatory bowel disease). Although this pathology involves multiple genes and environmental conditions, nod2 is present in the locus most strongly associated with the disease. Most mutations found in patients affect the LRR of the molecule, such as the frameshift mutation, the most common nod2 mutation associated with the disease. The frameshift mutation results in a protein unable to sense the natural ligand MDP [5,7], with impaired responses of macrophages from patients to MDP or MDP plus TLR agonists [7,25,26]. These observations are counterintuitive when considering an inflammatory disorder. However, recent studies might reconcile this apparent paradox. The nod2 mutations have been shown to lead to decreased antimicrobial activity in the gut and subsequently increased microflora proliferation. This might induce a loss of tolerance towards the commensal flora and abnormal inflammation [27].

Several mice models have been established to define further the role of Nod2 in IBD. Nod2-deficient mice displayed decreased antimicrobial peptides induction in a model of intra-gastric infection with Listeria, which correlates with the observations in humans [28]. However, the mice do not develop...
spontaneous intestinal inflammation. A knock-in transgenic mouse has been engineered, harbouring a mutation in Nod2 that mimics exactly the human nod2 frameshift mutation [29]. These mice had increased inflammation in a colitis model induced by dextran sodium sulfate. However macrophages from Nod2 frameshife mice still responded to the agonist MDP, showing a major difference with the human situation.

Nod2 is also implicated in the Blau syndrome [30]. Blau syndrome is a rare long-life disorder starting in childhood and characterized by skin rashes, uveitis and recurrent arthritis, which can evolve toward camptodactyly. The digestive tract is not involved in Blau syndrome, but, as in Crohn’s disease, granulomas associated with the inflammatory lesions are observed. The mutations in the nod2 gene found associated with this disease result in an overresponsive molecule, both constitutively and when triggered by MDP [30].

Interestingly, polymorphisms in the intronic region of nod1 have also been associated with IBD onset [31], even though these results were not supported by another study [32]. Another polymorphism in an intron of the nod1 gene has also been associated with susceptibility to asthma [33] and allergy [34]. It has been proposed that this polymorphism may affect the expression of specific nod1 splicing variants [33]. Since the corresponding isoforms of Nod1 displayed in vitro an impaired response to peptidoglycan [35], it is possible that defective detection of peptidoglycan by non-functional nod1 splicing variants may contribute to the onset of asthma.

Nod1 and Nod2 detect live bacteria

In addition to detecting peptidoglycan fragments (see above), Nod proteins have been shown to mediate responses to live bacteria. Nod1 has been shown to recognize Shigella flexneri [13], enteroinvasive Escherichia coli [36], Helicobacter pylori [37], Pseudomonas aeruginosa [38], Chlamydia spp. [39], Campylobacter jejuni [40] and Haemophilus influenzae [41]. Nod2 has been shown to sense Salmonella enterica [42], Listeria monocytogenes [28] and Streptococcus pneumoniae [43]. Interestingly, Nods seem to sense invasive and non-invasive bacteria, and, in the latter case, the stimulation of the intracellular Nod detection system has been shown to rely on a functional secretion system [37] or the presence of pore-forming toxins [41].

Activation of Nods induces antimicrobial factors

Epithelial cells are capable of producing antimicrobial peptides that control bacterial growth, and Nod1 and Nod2 are expressed in epithelial cell lines [42,44,45]. Nod1 seems to be more ubiquitously and constitutively expressed in this cell type than Nod2, even though several studies showed the up-regulation of Nod2 by TNFα and IFNγ (interferon γ) in epithelial cells [45,46].

Antimicrobial peptides represent the central component of the immune response in insects and in mammals, and display a large spectrum of microbicidal activities [47]. In vitro, various epithelial cell lines produce antimicrobial peptides following Nod1 [48] and Nod2 [49] stimulation. A recent study has demonstrated that Nod1 plays a key role in the induction of hBD-2 following infection of gastric cells with H. pylori [50]. Similarly, sensing of C. jejuni by Nod1 was shown to induce hBD2 production, resulting in lowered bacterial colonization [40]. In addition, the increased susceptibility of Nod2-deficient mice to oral infection with Listeria has been suggested to result from impaired production of cryptins, a family of antimicrobial peptides produced in the intestine [28].

NO (nitric oxide) is a compound that is known to be directly microbical, and its production depends on the induction of the iNOS (inducible NO synthase). iNOS is induced after Nod1 stimulation in mesothelial cells in synergy with IFNγ [51]. In primary macrophages, Nod1 [52] and Nod2 [51,53] pathways have been shown to induce the formation of NO.

Nod proteins induce inflammatory responses

Non-myeloid cells

Infection of epithelial cells with enteroinvasive E. coli [36] or H. pylori [37] results in the Nod1-dependent release of IL-8 (CXCL8), a chemokine critically implicated in neutrophil recruitment. In addition, exposure of endothelial cells to L. monocytogenes triggers Nod1-dependent NF-κB activation and IL-8 secretion [34].

Myeloid cells

Studies have implicated Nod1 and Nod2 in the release of cytokines from primary macrophages and DCs (dendritic cells). Although the levels are relatively low in most cases compared with the TLR-dependent stimulation, Nod ligands induce the release of several cytokines such as IL-8, TNFα, IL-6 and IL-1β (reviewed in [2]). In the case of IL-1β, it has been proposed that, upon MDP stimulation, Nod2 interacts with the molecular complex responsible for pro-IL-1β processing [55,56].

Importantly, the Nod ligands work in synergy with TLR agonists to induce greater amounts of inflammatory cytokines [57–59]. The mechanism of this synergy has yet not been clearly characterized, but it represents a more physiological situation when considering infections by whole live bacteria.

Direct injection of a Nod1 ligand into the mouse peritoneal compartment resulted in an important release of KC (keratinocyte cytokine)/CXCL1, IL-6 and TNFα, which are detected in the bloodstream 2 h post-injection [60]. As a result, resident peritoneal macrophages displayed enhanced expression of maturation markers, such as co-stimulatory molecules, at their surface. Accordingly, using similar procedures, a study demonstrated that Nod1-mediated cytokine release drives the recruitment of neutrophils in the peritoneal cavity [61].

Finally, Nod agonists have been established as an inducer of RANTES (regulated upon activation, normal T-cell expressed and secreted)/CCL5 secretion in primary mouse macrophages and in vivo, showing the chemotactic potential of the Nod pathways towards cell types other than
Nod1 and Nod2 link innate and adaptive responses

In addition to innate immune signals enhancing inflammation and phagocytosis via the recruitment of neutrophils, macrophages and DCs, Nod1 and Nod2 have been implicated in the induction of the adaptive immune responses. In human primary DCs, nod2 mutation produced reduced levels of IL-12 and failed to up-regulate the co-stimulatory molecules CD80 and CD86 in response to MDP plus TLR agonists [64].

MDP, the Nod2 agonist, has been known for more than three decades to be a potent adjuvant. As expected, the adjuvant effect of MDP was found to be totally dependent on Nod2 [28]. In this study, the predominant isotype induced by MDP as an adjuvant was the IgG1 (even after a boost immunization), which reflects a predominant Th2-type response [28]. In contrast, the stimulation of the primary DCs with MDP and TLR ligands resulted in the induction of IL-12, indicative of a Th1-type of response [28].

More recently, Nod1 has also been shown to play a key role in the induction of the adaptive response [65]. The injection of the antigen ovalbumin together with the Nod1 agonist FK156 as an adjuvant resulted, similarly to Nod2, in a Th2-biased immunity. Moreover, when the adjuvant was CFA (complete Freund’s adjuvant), which contains a mixture of nod and TLR ligands, the overall immune response was Th1-biased [65]. Nod1 deficiency was sufficient to blunt responses to adjuvant signals to CFA, suggesting that Nod1 plays a central role in the control of adaptive immune responses to bacterial preparations given as an adjuvant. Finally, chimaeric mice generated by transfer of bone marrow into lethally irradiated recipients were injected with CFA plus ovalbumin. Strikingly, non-myeloid cells were shown to be central in Nod1-mediated induction of immunity to the antigen [65]. These findings confirm and develop further a rising concept that epithelial and other stromal cells contribute to the onset of the adaptive response and influence it by the release of factors such as MCP1 (monocyte chemotactic protein 1) or TSLP (thymic stromal lymphopoietin).

Open question: why do we have Nod1 and Nod2?

Several orthologues of Nod1 and Nod2 are found in mammals, birds, fish and amphibians, but vertebrate species in which only Nod1 or only Nod2 is present have not been reported. This strongly suggests that Nod1 and Nod2 must have unique functions that do not overlap. This is supported further by the fact that Nod1 cannot compensate for Nod2 deficiency in Crohn's disease patients, and that Nod1- and Nod2-deficient animals display susceptibility in experimental models of infection to *H. pylori* and *Listeria* respectively. However, at first glance, a number of the respective functions of Nod1 and Nod2 seem to be common: both detect peptidoglycan intracellularly, induce NF-κB-dependent signalling and turn on the production of cytokines, chemokines and antimicrobial factors. The identification of the unique and irreplaceable functions of Nod1 and Nod2 represents a fundamental question for the coming years.

References


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