**NOD2: Activation During Bacterial and Viral Infections, Polymorphisms and Potential as Therapeutic Target**

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**ABSTRACT**

Nucleotide-binding domain (NBD) leucine-rich repeat (LRR)-containing receptors or NLRs are a family of receptors that detect both, molecules associated to pathogens and alarmins, and are located mainly in the cytoplasm. NOD2 belongs to the NLR family and is a dynamic receptor capable of interacting with multiple proteins and modulate immune responses in a stimuli-dependent manner. The experimental evidence shows that interaction between NOD2 structural domains and the effector proteins shape the overall response against bacterial or viral infections. Other reports have focused on the importance of NOD2 not only in infection but also in maintaining tissue homeostasis. However, not only protein interactions relate to function but also certain polymorphisms in the gene that encodes NOD2 have been associated with inflammatory diseases, such as Crohn’s disease. Here, we review the importance and general characteristics of NOD2, discussing its participation in infections caused by bacteria and viruses as well as its interaction with other pathogen recognition receptors or effectors to induce antibacterial and antiviral responses. Finally, the role of NOD2 in chronic inflammatory conditions and its potential to be targeted therapeutically are examined.

**Key words:** NOD2. Inflammasome. Infection. Polymorphisms. Inflammatory disease.

**INTRODUCTION**

The repertoire of pathogen recognition receptors (PRRs) in the innate immune system is encoded in the germ line. These PRRs are activated either by evolutionarily conserved pathogen-associated molecular patterns (PAMPs) or by endogenous damage-associated molecular patterns (DAMPs). PAMPs are molecules present in pathogens; in bacteria, they are found in the cell wall, flagella, lipoproteins, and...
nucleic acids. Furthermore, some cell wall components in fungi, as well as nucleic acids and proteins in viruses, are recognized as PAMPs. Unlike PAMPs, DAMPs are endogenous molecules released from cells under stress damage; these events are capable of initiating an inflammatory process. Some well-characterized DAMPs include DNA-binding proteins such as high-mobility group B1 proteins, heat-shock proteins, extracellular adenosine triphosphate (ATP), and uric acid crystals, among others.

PRRs are grouped into five families according to their structural domains (Fig. 1): (1) Toll-like receptors (TLRs), (2) retinoic acid-inducible gene-I-like receptors (RLRs), (3) C-type lectin receptors (CLRs), (4) absent in melanoma 2 (AIM2)-like receptors (ALRs), and (5) NOD-like receptors (NLRs). Following is a brief description of these PRRs.

TLRs are transmembrane glycoproteins that express an N-terminal ectodomain containing leucine-rich repeats (LRRs). Toll/interleukin-1 receptor (TIR) domain at their C-terminus allows signal transduction. TLR interaction with its ligand enables TIR domain dimerization and triggers signal transduction through TIR-domain-containing adapter-inducing interferon-β or myeloid differentiation factor 88 (MyD88). This signaling activates the nuclear factor κB (NF-κB) and induces the transcription of genes related to proinflammatory cytokines.

RLRs are a family of receptors that include molecules of the RIG-I and MDA-5 (melanoma differentiation-associated gene-5). RIG-I and MDA-5 display two N-terminal caspase activation and recruitment domains (CARDs) in tandem, a central DExD/H-box domain consisting of two helicase domains (Hel-1 and Hel-2), and a C-terminal regulatory domain. These receptors are cytoplasmic and detect viral RNA both single-stranded (ssRNA) and double-stranded (dsRNA).

CLRs contain three types of receptors. Type I receptors are transmembrane and contain several carbohydrate recognition domains (CRDs) (e.g., CD205 or DEC205, and macrophage mannose receptor 1 or
MMR-1). Type II receptors are transmembrane proteins and typically contain only one CRD (e.g., DC-SIGN and Dectin-1 or -2). The third type has a soluble form and includes the mannose-binding lectin (MBL). In these CLRs, two conserved domains confer specificity. While EPN (Glu-Pro-Asn) motifs confer specificity to mannose, QPD (Gln-Pro-Asp) motifs have other CRDs.

ALRs are cytoplasmic and express an N-terminal pyrin domain (PYD) that binds to DNA. After binding, the PYD domain interacts with another PYD domain in the adapter protein ASC (apoptosis-associated speck-like protein that contains a CARD). Then, ASC recruits and activates procaspase 1, and releases caspase-1 that processes the inactive precursors of interleukin-1β (IL-1β) into the mature form. This process induces an inflammatory form of cell death called pyroptosis.

NLRs are receptors located mainly in the cytoplasm but also in mitochondria and express one of the four possible binding domains at the N-terminal. According to these domains, we identify four subfamilies: (1) NLRA (A for acidic transactivating domain, e.g., CIITA), (2) NLRB (B for BIR, or baculovirus inhibitor of apoptosis protein repeat, e.g., NAIP), (3) NLRC (C for CARD, e.g., NOD2 or NLRC2), and (4) NLRP (P for PYD, e.g., NLRP3). In addition, these receptors contain an intermediate nucleotide-binding domain (NBD) also known as nucleotide oligomerization domain (NOD), which is necessary for binding and self-oligomerization. Finally, there is an LRR domain at the C-terminus. The best-characterized function of NLRs is to sense PAMPs or DAMPs and activate the immune response.

NLRs

The family of NLRs consists of 22 receptors in humans and 34 in mice. In humans, only eight of these have been well-characterized (Fig. 2). NLRs express one of the three structural domains (CARD, PYD, or BIR motifs) at the N-terminal which are involved in protein-protein interactions. Importantly, an intermediate NBD allows ATP binding and oligomerization. The C-terminal region in NLRs contains an LRR-domain similar to the one present in TLRs. The activation of some NLRs, such as NLRC4, NLRP1, NLRP3, NLRP6, NLRP7, and NLRP12, stimulates the inflammasome, which is a multimeric protein complex that mediates the activation of inflammatory caspases. Inflammasome activation leads the processing of caspase-1 and -11 in mice and -4 and -5 in humans, which generates the active forms of IL-1β, IL-18, and IL-33. Besides the processing of cytokines, these caspases contribute to the cleavage of gasdermin-D, which induces a particular type of cell death termed pyroptosis.

Despite their central role in activating the inflammasome, NLRs have some other very important regulatory activities. For example, NOD2 and NLRP3 regulate the signaling pathways of innate immunity, including the canonical and non-canonical pathways of NF-κB, mitogen-activated protein kinase (MAPK), and type I interferons (IFNs). They also regulate pathways that lead to the production of cytokines, chemokines, reactive oxygen species, and to the activation of ribonuclease L1. NOD2 and NLRC4, along with its coreceptor NAIP5, control the induction of autophagy and mitophagy. Meanwhile, NLRP10 and NLRC5 regulate and modulate another NLRs or major histocompatibility complex (MHC) genes through some molecules such as Class II, MHC, transactivator (CIITA). Finally, NLRP2 and NLRP7 participate in embryonic/fetal development and uterine implantation, which are required to control the immune response.

It is undeniable that NLRs and their multiprotein complexes participate in inflammasome-dependent as well as inflammasome-independent pathways. However, there is increasing evidence of their importance as the central effector of the immune response. A novel connection between endoplasmic reticulum stress and triggering of the innate immunity mediated by NOD1 and NOD2 has been described. The present review focuses on the functions of one NLR, the NOD2, during bacterial and viral infections, as well as on polymorphisms associated with disease susceptibility during infections and other pathogenic conditions. Moreover, the potential of NOD2 as a therapeutic target in the near future is exposed.

NOD2 DURING BACTERIAL AND VIRAL INFECTIONS

The NOD2 receptor has a molecular weight of 110 kDa (1040 aa) (Fig. 3). It expresses two CARD domains in the N-terminus, a central NBD, and an LRR
Figure 2. NOD-like receptors (NLRs): General characteristics and functions for the eight best-characterized NLRs in humans. Some NLRs received a name according with the domain present in their amino-terminal, such as NLRCs that present a caspase-activation and recruitment domain (CARD) and NLRPs having with a Pyrin domain (PYD). Functions of these NLRs include the formation of inflammasome and nodosome (axis NOD2/RIP2), and some of them exhibit regulator activities.

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Figure 3. Protein interactions established by the cytoplasmic receptor NOD2 relevant for immune responses. NOD2 is a very dynamic receptor; once it is activated in the cells, numerous interactions and responses are mediated. NOD2 is involved in a broad range of cellular responses that include inflammasome regulation, production of proinflammatory cytokines, triggering autophagy, production of type I interferons, and other antiviral activities such the activation of RNAse L.
domain at the C-terminus\textsuperscript{16}. NOD2 is able to detect muramyl dipeptide (MDP), a molecular motif commonly expressed in the peptidoglycan (PGN) of Gram-negative and Gram-positive bacteria. This receptor is commonly expressed in cells that include Paneth intestinal cells, monocytes/macrophages (Mo/M\textsubscript{φ}s), dendritic cells (DCs), and granulocytes.

NOD2 is predominantly located in the cell cytoplasm and is able to interact with multiple proteins (Fig. 3). Following increased expression of protein \textit{in vitro}, NOD2 becomes associated with the plasmatic membrane, an event that seems important to activate NF-κB\textsuperscript{37}. M\textsubscript{φ}s treated with MDP exhibit NOD2 molecule in their acidic vesicles\textsuperscript{1}. Several studies on M\textsubscript{φ}s indicate that while DCs from animals and humans show that stimulation of NOD2 by MDP induces a variety of responses that clearly differ from those triggered by TLRs, the activation of NOD2 can, in fact, induce a two- to three-fold potentiation of the response mediated by TLRs\textsuperscript{18}.

NOD2 enables those TLRs localized in the cytoplasmic membrane to complete or increase their response. This is particularly important in infections caused by intracellular pathogens\textsuperscript{16}. For example, after the recognition of MDP by NOD2, and LPS by TLR4, the activation of both PRRs is synergistic and cytokine production is significantly potentiated\textsuperscript{19}. An analysis of transcripts from cells infected with vacuolar pathogens showed a TLR response dependent on induction of MyD88. Meanwhile, in cells infected with intracellular pathogens, a NOD2- and interferon regulatory factor 3 (IRF3)-dependent responses were found\textsuperscript{20}.

Various authors have reported the involvement of NOD2 in bacterial infections (Fig. 4). NOD2 also participates in the expression of the inducible nitric oxide (NO) synthase to produce NO during \textit{Mycobacterium tuberculosis} infections of human M\textsubscript{φ}s\textsuperscript{21}. NOD2 mRNA levels increased in a rat model for \textit{Staphylococcus aureus}-induced mastitis\textsuperscript{22}, while NOD2 mRNA, as well as protein levels, increased in the central nervous system of a mouse model for pneumococcal meningitis\textsuperscript{23}. NOD2 has also been reported to participate in the immune response to periodontal pathogens\textsuperscript{24,25} and contributes to the bone loss mediated by \textit{Porphyromonas gingivalis}\textsuperscript{25}. In cells infected with extracellular and intracellular bacteria, there is an increase in the expression and activation of NOD2, based on dependent and independent recruitment of receptor-interacting protein 2 (RIP2). Apart from this significant interaction of NOD2 with RIP2, other non-canonical signaling pathways are also affected. Three of the most important interactions occurring with NOD2 during infections are herein described.

The response of NOD2 to MDP initiates a signaling cascade activating NF-κB and MAPK in a TLR-independent manner. Thus, NOD2 senses MDP through its LRR domain which then induces the unfolding of the NBD domain, followed by self-oligomerization, and exposure of its CARD domains\textsuperscript{1,16}. These events lead to the recruitment and binding of RIP2\textsuperscript{26} by homophilic interactions through CARD-CARD\textsuperscript{27}. The binding of NOD2 and RIP2 culminates in the activation of NF-κB. RIP2 molecule recruits TNF receptor-associated factor 6-E3 ubiquitin-protein ligase, which subsequently triggers self-ubiquitination. The latter also has the ability to polyubiquitinate other proteins downstream from NOD2\textsuperscript{26}. The kinase domain of RIP2 is associated with other E3 ubiquitin ligases such as cellular inhibitors of apoptosis (cIAP1/2). Both catalyze the ubiquitination of RIP2 at the Lys63 site (K63). The polyubiquitination of RIP2 recruits the transforming growth factor beta-activated kinase 1 complex (TAK1) (TAK1-TAB1/2/3), leading to the activation of the IkK kinases\textsuperscript{29}. The phosphorylation and recruitment of the IkKβ kinase inhibit NF-κB through degradation of IκBs by the proteasome\textsuperscript{1,27} (Fig. 4).

CARD9 is expressed predominantly in DCs and M\textsubscript{φ}s; consequently, it is found in lymphoid organs and is an adapter protein with a CARD domain at the N-terminus and a “coiled-coil” domain at the C-terminus. It regulates signaling during fungal infections and is required in the immune response against intracellular pathogens\textsuperscript{30}. In M\textsubscript{φ}s and DCs treated with PGN, NOD2 interacts with CARD9; this interaction activates p38 extracellular signal-regulated kinase (ERK)\textsuperscript{1} and c-Jun N-terminal kinase (JNK), which activates the heterodimeric transcription factor activator protein 1\textsuperscript{29}. The M\textsubscript{φ}s of mice deficient in Card9-/- express defects in the activation of p38 and JNK kinases after a viral and bacterial infection, but not in NF-κB. Whereas the overexpression of CARD9 and NOD2 normally occurs during infection with \textit{Listeria monocytogenes}, in CARD9-/- mice cytokine production is deficient, and this bacterium cannot be eliminated\textsuperscript{30}.  


Autophagy is a highly conserved catabolic process of eukaryotic cells used to recycle macromolecules. This occurs in double-membrane vesicles that sequester damaged organelles, which are degraded afterward by the fusion of lysosomes to these vesicles. Membrane-recruited NOD2 participates in the formation of autophagosome by interacting with the autophagy-related 16-like 1 protein complex (ATG16L1). During the bacterial invasion, ATG16L1 and NOD2 lead bacteria elimination. Although it is known to be an NF-κB-independent mechanism, the involvement of RIP2 is still being debated (Fig. 4). The Mos of TLR2−/−, NOD2−/−, and RIP2−/− mice are more susceptible to the infection with *L. monocytogenes*. In this infection model, the activation of the axis NOD2/ RIP2 and the consequent induction of autophagy is dependent on the ATG16L protein, and the ERK-signaling pathway. Thus, the protective response to *L. monocytogenes* depends on TLR2 and NOD2 signaling. In infections with *Salmonella enterica* serovar typhimurium, the induction of autophagy by NOD2 is necessary for the antigen presentation to take place in DCs.

NOD2 stimulation with viral ssRNA activates the interferon regulatory factors 3 and 7 (IRF3 and IRF7) and induces an antiviral response mediated by type I IFNs. This has raised new questions about molecular recognition and signaling by NOD2 because there are no structural similarities between bacterial MDP and viral ssRNA or dsRNA motifs. NOD2 along with other NLRs, such as NOD1, might promote inflammation and facilitate antiviral response. It is plausible that the activation of NOD2 occurs following a direct interaction with viral genome or proteins during the viral replication cycle.
NOD2 activation has been demonstrated in several infections with viruses (Fig. 5) that have an ssRNA genome such as vesicular stomatitis virus, respiratory syncytial virus (RSV), and parainfluenza virus 3, and also for some viruses with DNA genomes such as human cytomegalovirus. In these infections, there is an increased expression of NOD2 and type I IFNs in human bronchial epithelial cells, MΦs, and embryonic fibroblasts. In MΦs and epithelial cells NOD2 binds to other proteins apart from RIP2 including CARD9, ATG16L1, mitochondrial antiviral-signaling protein (MAVS), and 2'-5'-oligoadenylate synthetase type 2 (OAS2) (Fig. 5).

A study of neutrophils from individuals exposed to human immunodeficiency virus revealed that the hyporesponse of NOD2 and other effectors are related to the maintenance of seronegativity.

Recent evidence suggests that leukotriene B4 directly impacts on the NOD2 pathway enhancing the immune response against influenza A virus (IAV). On the other hand, hepatitis E virus exhibits an intrinsic ability to counteract the activity of NOD2 and other PRRs.

During viral infections, an activation of the canonical axis NOD2/RIP2 also occurs. In an infection model of NOD2−/− and RIP2−/− mice with IAV, these become hypersensitive to the infection. The analysis of individual of RIP2−/− cells showed that this is due to the induction of mitophagy (mitochondrial autophagy), a phenomenon that causes an increase in superoxide production and leads to mitochondrial damage. This causes a strong activation of the NLRP3 inflammasome, resulting in increased production of IL-18. The RIP2 protein regulates mitophagy through
phosphorylation of the Unc-51-like kinase 1. This
model demonstrates that NOD2 and RIP2 downregu-
late the activation of the NLRP3 inflammasome and
production of IL-18 through ULK-1.41

In addition, during viral infections, NOD2 can also be
relocated to the mitochondria by its interaction with
the MAVS protein through its LRR and NOD domains.
This interaction triggers the nuclear translocation
of IRF3 and induces the production of type I IFNs26,28
(Fig. 5). The activation mechanism involves macromo-
lecular complexes consisting of RIP2 and TRAF3.28,29
In fact, depletion of NOD2 abates the expression
of type I IFNs. Viral ssRNAs may activate the NOD2
receptor, causing activation of the MAVS protein in the
mitochondria. MAVS phosphorylates IRF3/IRF7, two
factors that form homodimers and translocate to the
nucleus.26,36 Moreover, the interaction of NOD2 and
the adapter protein MAVS regulate the production of
type I IFN and increase expression of NOD2 de novo. It
is possible that NOD2 interacts with the MAVS-RIG-I/
MDA-5 complex associated with viral RNA.11

In a murine model of infection with RSV, the activation
of NOD2 allows its relocation to the mitochondria and
increases NOD2 mRNA expression coinciding with the
increased expression of RIG-I and TLR3, suggesting the
synergic role of the latter two receptors in the antivi-
ral response.37 Proteomic analysis revealed that NOD2
interacts with OAS2 in the THP-1 cell line.42 The OAS2
molecule is necessary for the activation of RNase L,
which degrades viral and cellular RNA limiting viral
replication.14,51 The interaction between NOD2 and
OAS2 takes place during cellular response to dsRNA
and synthetic ligands such as poly (I:C), which mimic
viral RNAs. Data suggest that in some viral infections,
NOD2 possibly participates in inducing the expression
of type I IFNs through a mechanism independent of
RLRs26. Clearly, NOD2 is a dynamic cytoplasmic recep-
tor exhibiting an exceptional plasticity, whose cellular
activity depends on the adapter molecules recruited in
each response.26,37

**NOD2 POLYMORPHISMS INVOLVED IN
HEALTH AND DISEASE**

NOD2 deficiency in mice has been associated with
chronic inflammatory disease. In humans, a mutation
in the CARD15 gene (in the LRR region of NOD2) has
been associated with chronic bowel inflammation in
Crohn’s disease (CD). Moreover, polymorphisms in this
receptor are associated with various diseases such as
Blau syndrome, arthritis, atopic dermatitis, sarcoid-
odis, and possibly asthma.43,44

The gene encoding NOD2 is located in the “q” region
of chromosome 16 and is highly polymorphic (Fig. 6),
with at least 660 single nucleotide polymorphisms
(SNPs) described with alleles varying among individ-
ual populations and across geographical locations.
There are three main polymorphisms in NOD2 fre-
quently associated with disease, SNP8 (rs2066844),
SNP12 (rs2066845), and SNP13 (rs41450053)45.
Individuals that are heterozygous for any of these
SNPs show a 2- to 4-fold greater risk of developing
CD, while those homozygous for these SNPs exhibit
an almost 20-fold greater risk of developing this
disease.46,47

SNPs 8, 12, and 13 in the NOD2 gene are located in
exons 4, 8, and 11, respectively. Whereas there is
only an amino acid change in SNP8 and 12, a frame-
shift takes place in SNP13, leading to the emergence
of a truncated protein.48 Some polymorphisms in the
region coding for NOD2/CARD15 increase the risk of
developing CD up to 17.1-fold in either homozygous
or heterozygous individuals.49 Mutations in NOD2/
CARD15 decrease activity in the pathway, leading
to inhibition of NF-κB. This causes the over-reactiva-
tion of this protein complex and the proinflammatory
symptoms observed in CD.50

In addition to CD, these polymorphisms in NOD2 have
been implicated in other ailments such as anquilosante
spondylitis, arthritis, and cancer.10 Moreover, NOD2
(rs8057431) seems to be implicated in susceptibil-
ity to Mycobacterium leprae in diverse populations.51
Furthermore, the SNP13 in NOD2 has been implicated
in septic shock following transplantation with stem
cells.52

Strikingly, NOD2 sensing activity of commensals has
been shown to be indispensable to aid in the right sort-
ing of antimicrobial peptides to the intracellular com-
partments of the Paneth cells and also for the correct
establishment of symbiosis.53 Thus, in CD associated
with NOD2 deficiencies, the ability to respond to the
MDP present in commensal and pathogenic bacteria is
clearly altered.54
IS NOD2 A SUITABLE THERAPEUTIC TARGET?

Current data on NLRs suggest that these molecules hold a central role in the innate immune response as well as in regulating proinflammatory pathways. In 2008, for example, two independent studies reported that NLRP3 is a chief sensor in response to aluminum salts\textsuperscript{55,56}, which are the lone approved adjuvant for human vaccines. This represents the first insight into the action mechanisms for these salts. Stimulation of NOD2 by MDP triggers a wide repertoire of transcripts in vitro, including those encoding for chemokines, proinflammatory cytokines, antimicrobial peptides, and adhesion molecules\textsuperscript{57}. Several authors have demonstrated that resistance to viruses is conferred by MDP alone or in combination with other agents. As MDP signaling is mostly carried out through NOD2, this NLR has been envisioned as a potential therapeutic target\textsuperscript{58}.

Considering that NOD2-mediated inflammation exacerbates some ailments, it could be a suitable therapeutic target through its downregulation. An important point of intervention is through the RIP2 molecule, which as mentioned is downstream of the NOD2 signaling pathway. RIP2 activity can be effectively impeded by existing pharmaceutical type II kinase inhibitors\textsuperscript{59}.

CONCLUDING REMARKS

NOD2, a PRR, exhibits critical activities during bacterial and viral infections. NOD2 is able to synergize with TLRs and has an important role in the activation of NF-\textkappaB. Moreover, due to its dynamic nature, NOD2 can determine multiple cell responses not only in infectious diseases but also in health. Therefore, this molecule constitutes an important target for pathogen evasion mechanisms and is also a potential target to be intervened therapeutically in the future.

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