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Article type : Invited Review

Title: NOD1 and NOD2 in inflammatory and infectious diseases

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Running title: NOD1 and NOD2 signaling and function

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/IMR.12902

3-6 keywords: Innate, immunity, Nod-like receptor, NOD1, NOD2, inflammation

Summary

It has been long recognized that NOD1 and NOD2 are critical players in the host immune response, primarily by their sensing bacterial peptidoglycan-conserved motifs. Significant advances have been made from efforts characterize their upstream activators, assembly of signaling complexes, and activation of downstream signaling pathways. Disruption in NOD1 and NOD2 signaling have also been associated with impaired host defense and resistance to the development of inflammatory diseases. In this review, we will describe how NOD1 and NOD2 sense microbes and cellular stress to regulate host responses that can affect disease pathogenesis and outcomes.

Introduction

Pattern recognition receptors (PRRs) have a central role in regulating immune and inflammatory responses, and the magnitude of their responses can affect the outcome of infections, autoimmune and inflammatory diseases, and cancer risk. Amongst the most well-characterized PRRs are the nucleotide-biding oligomerization domain (NOD)-like receptors (NLRs). The NLRs are cytosolic receptors which sense microbial motifs, endogenous byproducts of tissue injury and environmental signals; however, mechanisms by which each NLR member recognizes different signals are still not well-understood. Once activated, they mediate various effector functions via signal transduction pathways that regulate such processes as cellular death and proliferation, autophagy, tissue repair, and inflammation. Consequently, NLRs are important in maintaining tissue and immune homeostasis that when dysregulated can lead to inflammatory diseases. NOD1 and NOD2 are two seminal NLRs that have been shown to sense specific bacterial ligands and have diverse immunomodulatory effects that are important for both host defense and tissue homeostasis. In this review, we will describe host responses regulated by NOD1 and NOD2 and their impact on host-microbial interactions and inflammatory disease pathogenesis.

Structure of NOD1 and NOD2 receptors

NLRs are characterized by a tripartite domain structure consisting of a C-terminal leucine rich repeat (LRR) domain required for ligand sensing, a central long nucleotide-binding NACHT domain (NBD domain) that mediates oligomerization, and a variable N-terminal effector domain important for interactions with downstream effector proteins. NLRs are further divided into four subfamilies based on the nature of their N-terminal domain: 1) NLRP contain a pyrin domain (PYD), 2) NLRA contain an acidic transcriptional activation (TA) domain, 3) NLRB have a baculovirus IAP repeat (BIR) domain, and 4) NLRC have a caspase activation and recruitment domain (CARD), respectively. As founding members of the NLRs, NOD1 and NOD2 have often retained their original nomenclature; however, since they contain an N-terminal CARD domain, they belong to the NLRC subfamily of NLRs, and are technically designated as NLRC1 and NLRC2, respectively (1). NOD1 and NOD2 have similar domain architectures, but differ in the number of CARD domains: NOD1 contains one, whereas two tandem CARD domains are found in NOD2 (*Figure 1*). Systematic mutagenesis experiments have been informative in delineating the functions of the various domains in the activity of NOD1 and NOD2. The CARD domain is critical for interaction with the downstream adaptor protein RIP2 with crucial residues in both CARD domains of Nod2 important for this activity (2-4)(5). Within the centrally-located NBD are Walker A- and B- box motifs that contain residues important for ATP binding and hydrolysis as well as ligand-dependent activation of downstream signaling pathways, notably NF-κB (5, 6). ATP binding by NOD2 enhances both ligand binding and oligomerization, and mutations in this region that disrupt this can abrogate downstream signaling (6, 7). On the other hand, inhibition of ATP hydrolysis and stabilization of binding results increased activity. Interestingly, mutations in a highly conserved patch of acidic residues (extended Walker B box) in NOD2 that have been associated with autoinflammatory disease such as early-onset sarcoidosis and Blau syndrome, results in constitutive activation of NOD2, but inactivation of NOD1, suggesting that despite strong similarities in domain structure, different mechanisms are involved in their activation (6).

The C-terminal LRR domain differs in size between NOD1 and NOD2 and is crucial for ligand recognition and binding (8, 9). Comparative genomic studies show high

conservation particularly within the LRR among different species in residues especially along a predicted concave surface of the LRRs to form a ligand binding site (10), which is also consistent with the crystal structure of NOD2 (11). Furthermore, the LRR is solely responsible for dictating ligand specificity (5, 8).

Activators of NOD1 and NOD2

NOD1 and NOD2 recognize distinct fragments of peptidoglycan (PGN) that is a major component of the bacterial cell wall of Gram-positive bacteria, outside of the cytoplasmic membrane, providing them structure, rigidity, and protection (12, 13). A thin layer of PGN is also found in Gram-negative bacteria within the periplasmic space. PGN consists of a polymeric chain of alternating sugar residues of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) that form the backbone with short peptides covalently bound to MurNAc to create a muramyl peptide, which can be crosslinked to form the lattice structure of peptidoglycan (14) (*Figure 2*). The short peptide chains in the PGN contain 3-5 amino acids that are differentially found in Gram-negative and Gram-positive bacteria and are also differentially recognized by NOD1 and NOD2 (14). Muropeptides are peptidoglycan fragments that can be generated by degradation of PGN either by host or bacterial enzymes. NOD1 senses muropeptides containing the minimal γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) dipeptide core, which is predominantly found in Gram-negative, but also in a few Gram-positive bacteria such as Listeria monocytogenes, and Bacillus spp (15, 16). Interestingly, screening of bacteria derived from soil revealed that the genus *Bacillus* bacteria has the strongest NOD1-stimulatory effect associated with culture supernatants rather than bacterial cell extracts, and are also highly stable, more so than Toll-like receptor 4 (TLR4) or NOD2 ligands, suggesting the potential for environmental stimuli to contribute to homeostatic regulation of the immune system and that NOD1 agonists can be produced and released by bacteria to activate NOD1 (17). Indeed, muramyl peptides spontaneously shed by the Shigella are able to trigger a NOD1 mediated response, and *Listeria* lacking streptolysin O, which is required to escape from the phagosome into the cytosol, is still able to activate NOD1 (17, 18). Furthermore, the synthetic addition of lipophilic acyl residues greatly enhanced Nod1 activation and may be related to interaction with the cell membrane to facilitate

translocation into the intracellular compartment of the host cell for recognition by NOD1 (19). Although a direct interaction between NOD1 and the ligand l-Ala-γ-D-Glu-meso-diaminopimelic acid (Tri-DAP) that is LRR-dependent has been demonstrated by surface plasmon resonance and atomic microscopy (20), it remains unclear whether this is true for all ligands and whether other cofactors are involved in ligand-binding.

In contrast to NOD1, NOD2 requires PGN fragments containing an intact MurNac ring structure and an attached sugar to the dipeptide moiety and has been shown to directly bind muramyl dipeptide (MDP) that is broadly expressed in both Gram-positive and Gram-negative bacteria (21-23). To activate NOD2, MDP may have an intact MurNAc ring structure, and the sugar must be attached to the peptide moiety (23). Unlike NOD1, the length of the peptide moiety is not critical for recognition, and therefore meso-DAP-muropeptides can be recognized by both NOD1 and NOD2, allowing NOD2 to be activated by a broader range of muropeptides from both Gram-negative and Gram-positive bacteria (14). Although several studies have demonstrated a direct interaction between NOD2 and MDP (7, 22, 24), it remains to be determined whether direct binding occurs with larger MDP-containing PGN fragments and whether additional accessory proteins are involved. For example, it has been shown that glucosaminyl-MDP is capable of binding to YB1, which can form a complex with NOD2 leading to cooperative activation of downstream signaling pathways (25).

There is some evidence to suggest that in addition to bacterial peptidoglycan fragments, NOD1 and NOD2 can recognize other types of ligands such as viral RNA. Both *in vitro* experiments with overexpressed proteins and *in vivo* studies using *Nod2*-deficient mice demonstrated the ability of viral single-stranded RNA (ssRNA) to activate NOD2 that is dependent on the presence of both the NBD and LRR domains. However, ssRNA is unable to activate NOD1 (26). Rather, at least *in vitro* in a hepatocyte cell line, synthetic dsRNA (polyI:C) or dsRNA generated by the RNA polymerase of Hepatitis C virus induces NOD1 expression and interacts with NOD1, resulting in activation of downstream signaling pathways (27). Interestingly this interaction occurred independently of the LRR, but requires an intact NBD for full activity (27). Similarly, zebrafish NOD1 overexpressed in the carp fish cell line epithelioma papulosum cyprinid (EPC) was capable of binding to the dsRNA virus, spring viremia of carp virus (SVCV),

as well as poly I:C that was CARD-dependent, suggesting perhaps the involvement of an intermediary protein for this interaction (28).

Besides pathogen-associated molecular patterns (PAMPs), there is increasing evidence that NOD1 and NOD2, like other NLRs, can also respond to danger signals, or damage-associated molecular patterns (DAMPs). Notably, disturbances in endoplasmic reticulum (ER) function can lead to ER stress with accumulation of misfolded proteins within the ER lumen, which if left unchecked, can lead to cellular injury and apoptosis. Thus, ER stress, which can be induced by various cellular stressors including microbial infection, can be construed as a danger signal indicative of cellular dysfunction. As a means of restoring ER function and cellular homeostasis, ER stress triggers the activation of various signaling pathways including inflammation, that constitute the unfolded protein response (UPR) (29). Chemical inducers of ER stress, such as thapsigargin and dithiothreitol resulted in upregulation of the inflammatory cytokine IL-6, which was in part dependent on the presence of NOD1 and NOD2 (30). In addition, infection with *B. abortus* which induces ER stress also promoted an inflammatory response in bone marrow-derived macrophages and in mice that was reversed by the ER stress inhibitor TUCDA and was impaired in the absence of NOD1 and NOD2. Together, these results strongly suggest a PGN-independent pathway involving ER stress can activate NOD1 and NOD2 although the precise upstream signal sensed by NOD1 and NOD2 remains unclear. However, it has also been posited based on results from another study that ER stress *per se* does not activate NOD1 and NOD2; rather, cellular perturbations related to Ca^{2+} flux may be the main activating stimulus as thapsigargin, which induces ER stress by inhibiting ER calcium ATPase and Ca^{2+} accumulation in the ER lumen, resulted in NOD1/2-dependent cytokine production in HCT116 colon epithelial cells (31). On the other hand, other ER inducers not associated with changes in intracellular Ca^{2+} levels, such as tunicamycin, did not induce an inflammatory response via NOD1 or NOD2 (31). Furthermore, inhibitors of Ca^{2+} flux partially recapitulated the effects of thapsigargin. Therefore, it was hypothesized that increases in intracellular Ca^{2+} can lead to endocytosis and internalization of extracellular PGN fragments present in either media and serum to activate NOD1 or NOD2 to explain results from previous studies although this has not been definitely proven (31)

PGN fragments from non-invasive bacteria can be transported into the eukaryotic cytosol through bacterial secretion systems where they are sensed by both NOD1 and NOD2 receptors. However, bacterial effector proteins belonging to the Type III secretion system can also activate NOD1 and NOD2 independently of PGN stimulation that may be related to perturbations in the cytoskeleton. In particular, NOD1 and NOD2 can sense the activation state of Rho GTPases, which regulate the actin cytoskeleton and various signal transduction pathways (32). For example, bacterial effector proteins such as the Salmonella Type III secretion system protein SopE, can act as a guanine nucleotide exchange factor and efficiently activate the Rho GTPases CDC42 and Rac1, which then interacts with NOD1 resulting in activation of downstream signaling pathways, specifically NF-κB (33, 34). More specifically, the presence of SopE introduces membrane ruffles and recruitment of NOD1 into a multiprotein complex containing SopE, Rac1, CDC42, Hsp90, and NOD1. SopE containing a mutation that is unable to activate Rac1 or CDC42 did not result in membrane ruffling and recruitment of NOD1. Similarly, *Salmonella* SipA, another Type III secretion system effector protein, can colocalize with NOD1 and NOD2 and activate NF-κB in a NOD1- and NOD2-dependent manner, but independently of PGN (35). *Shigella flexneri* effector proteins, such as OspB and IpgB2, can also induce membrane ruffling resulting in recruitment of the guanidine exchange factor, GEF-H1, which, in turn, activates the rho GTPase, RhoA, leading to NF-κB activation that requires NOD1 (36). NOD2 can also interact with Rho GTPases; specifically, it was demonstrated that NOD2 can be co-immunoprecipitated together with Rac1 in HT29 colon epithelial cells with localization of NOD2 to membrane ruffles induced by active Rac1 (37). The ESX-1 bacterial secretion system of *Mycobacterium tuberculosis* can also cause pore formation and cell membrane damage, resulting in activation of NOD2 (38, 39). It is, therefore, possible that under these conditions, what activates NOD1 and NOD2 are disturbances in the actin cytoskeleton structure. Consistently, treatment of the HT29 colon epithelial cell line with cytochalaisan D resulted in NF-κB activation that required NOD2 in the absence of with PGN (37).

Cellular Localization of NOD1/NOD2

Although NOD1 and NOD2 are cytosolic receptors, their activation is associated with localization to the plasma membrane, bacteria containing phagosomes, and endosomes likely reflecting points of bacterial and ligand entry (40-43). Association with the plasma membrane by NOD1 or NOD2 also typically requires an intact LRR domain, and mutations in the LRR domain resulted in localization largely to the cytosol (42). How recruitment to intracellular membranes occurs remains to be fully elucidated, but may involve interaction with the actin cytoskeleton or via interactions with proteins that can associate with the membrane (37, 42, 43). For example, NOD2 colocalizes with vimentin on the cell membrane (44) and is also capable of binding to Erbin and FRMPD2 (45, 46), a negative and positive regulator of NOD2 signaling, respectively, on the basolateral membrane of intestinal epithelial cells. Rho GTPases, which regulate the actin cytoskeleton, are capable of interacting with NOD1 or NOD2 for their activation (33, 44-46). It was also demonstrated that recruitment to the plasma membrane requires lipid modifications for their recruitment to the cell membrane. Specifically, S-palmitoylation mediated by the ZDHHC5 enzyme, which has been associated with the endosomal system and is constitutively localized to phagosomes, was required for membrane recruitment to mount an effective immune response to PGN (47).

Mechanisms of ligand delivery

Given their intracellular location, efficient NOD1 and NOD2 activation require ligands to be delivered into the cytosol and entry be achieved by multiple routes (*Figure 3, Table 1*). Polymeric PGN undergoes phagocytosis and lysosomal digestion (48) and may, thereafter, be transported to the cytosol. Ligands can be internalized by host cells through phagocytosis of whole bacteria, endocytosis, and uptake of outer membrane vesicle (OMVs) (49).

There are multiple potential mechanisms by which PGN fragments can enter the cell to activate NOD1 and NOD2, such as bacterial phagocytosis as well as endocytosis dependent on clathrin and dynamin (50-52). Although certain bacterial secretion systems can activate NOD1 and NOD2 independent of PGN, delivery of PGN can also occur via bacterial secretion systems as is the case with *Helicobacter pylori*, whose detection by

NOD1 is dependent on an intact Type IV secretion system and delivery of PGN (53). Also, invasive bacteria, such as *Shigella flexneri*, shed ligands into the cytosol (18).

Membrane transport systems can also transport PGN fragments across the cellular surface into the cytosol. The peptide transporters of the SLC15 family were shown to mediate ligand delivery (51, 52). The delivery process occurs through either micropinocytosis in a G-protein-coupled calcium-sensing receptor (CaSR)-dependent manner or macropinocytosis in phagocytes (54). PepT1 (SLC15A1), which is highly expressed in the small intestinal epithelium as well as in the colon during inflammation, transports specifically MDP, but not NOD1-activating molecules (55, 56). On the other hand, PepT2 (SLC15A2), which is also expressed in epithelial cells, such as in the lung, was capable of actively transporting the NOD1 ligand ieDAP, but not MDP, to activate inflammatory responses in a NOD1-dependent manner (57, 58). The relative importance in the utilization of these transporters may also be dependent on cell type as PepT1, although important for transport of MDP into intestinal epithelial cells, was not required for MDP entry into macrophages, and similarly, transport of NOD1 ligands did not involve PepT2 in HEK293T cells (51, 52). Although PepT2 was not important for MDP entry into lung epithelial cells, there was reduced activation of NOD2 in response to NOD2 agonists, such as MDP in *Pept2*-deficient bone marrow macrophages (57, 59). Transport of NOD1 and NOD2 ligands out of endosomes are mediated by additional transporters, including SLC15A3 and SLC15A4 (Pht1) that are specifically expressed in macrophages and dendritic cells (41, 52, 59, 60). These studies demonstrate the importance of transporters for intracellular peptidoglycan delivery and activation of NOD1 and NOD2, but the events occurring downstream of peptide transportors remain to be fully elucidated.

Gram-negative bacteria can release outer membrane vesicles (OMVs) that are 10 to 300 nm in diameter and can contain PGN fragments, which can be internalized to activate NOD1 and NOD2. Both pathogens and commensal bacteria can produce OMVs, and may be a mechanism by which non-phagocytic cells, such as epithelial cells, internalize PGN to enable NOD1 or NOD2 signaling. Pathogens, such as *Vibrio cholerae, Helicobacter pylori, Pseudomonas aeruginosa, Neisseria gonorrhea, and Aggregatibacter Actinomycetemcomitans,* can produce PGN-containing OMVs that activate either NOD1 and/or NOD2 (61-63). Besides pathogenic bacteria, OMVs from

commensal bacteria such as the gut microbiota are also capable of activating NOD1 and NOD2 in intestinal epithelial cells (64). Although it was shown that OMVs can enter the host cytosol via lipid rafts located on the cell surface (62) and that NOD1 can colocalize with OMVs at early endosomes (40, 64), the precise mechanism of OMV entry into the cytosol and their localization within the cell can differ depending on the bacterial strain and host cell type (65, 66).

Signaling pathways downstream of NOD1/2.

NOD1 and NOD2 regulate multiple pathways involved in a variety of cellular responses, including inflammatory responses via activation of NF-κB, MAPK, and Type I IFNs, and autophagy (see *Figure 4*). In the absence of any stimulation, both NOD1 and NOD2 exist in an inactive "autoinhibited" state in which the LRR domain folds onto the NBD and CARD domain preventing oligomerization and engagement of proteins involved in downstream signaling pathways (see Figure 4) (11). Consistently deletion of the LRR domain or mutations that enforce oligomerization in NOD1 is sufficient to induce NF-κB activation (4, 67). Upon sensing their cognate ligands, NOD1 and NOD2 self-oligomerize via their NACHT domain to recruit and likewise bring into close proximity molecules of the adaptor protein receptor-interacting serine-threonine kinase 2 (RIPK2), which contains an N- and C-terminal CARD domain, via homotypic CARD-CARD interactions (4, 67)(3, 68-70). This subsequently leads to K63-linked polyubiquitination of RIP2 within its kinase domain, which is required for activation of NF-κB (71). Multiple E3-ligases are involved in the poly-ubiquitination of RIPK2 and include cIAP-TRAF complexes and Pellino3 (71-73) (71). Polyubiquitination of RIPK2 is necessary for recruitment of transforming growth factor B-activated kinase 1 (TAK1) and TAK1-binding proteins (TAB), which subsequently lead to MAPK and NF-κB activation. Non-K63 ubiquitination of NOD2 can also occur via XIAP and leads to the recruitment of the linear ubiquitin chain assembly complex (LUBAC), which produces M1-linked ubiquitin chains that together with K63-linked chains, facilitate the activation of NF-κB (74). On the other hand, deubiquination of RIPK2 by the deubiquitinases A20, OTULIN, or cylindromatosis protein (CYLD) results in downregulation of NOD1 and/or NOD2 signaling (75-78)(71, 79).

RIPK2 is critical for activation of both MAPK and NF-κb pathways. RIPK2 binds to both TAK1 and the NF-κB essential modulator kinase (NEMO) also known as IKKγ (71). The colocalization of NEMO and TAK1 promotes the subsequent phosphorylation of the IκKβ subunit of the inhibitor of KappaB Kinase (IKK)-complex by TAK1, which results in the phosphorylation and degradation of IκBα from the IKK-complex. The IKK-complex then drives phosphorylation of signal responsive serine residues of inhibitors of KappaB (IκBs), which are bound to NF-κB dimers in the cytosol. IκBs undergo proteosomal degradation to allow the cytoplasmic release and nuclear translocation of NF-κB followed by transcription upregulation of pro-inflammatory mediators (80). NF-κB subsequently binds to KappaB (κB)-elements, activating transcription of inflammatory and immune response components. Initiation of RIPK signaling and formation of the TAK1 kinase complex also results in phosphorylation of MKK6 and subsequent activation of the MAPKs, including the p38, extracellular signal–regulated protein kinase (ERK), and c-Jun N-terminal kinase (JNK) pathways (81)(69, 82-84). Phosphorylated MAPKs then translocate into the nucleus and phosphorylate AP-1 transcription factors, such as ATF, c-fos, c-Jun and JDP family members, and mediate cytokines, chemokines and antimicrobial peptide expression. Thus, the engagement of RIPK2 and TAK1 allows NOD1 and NOD2 to upregulate inflammatory responses via both NF-κB and MAPKs.

Formation of the NOD1/2-RIPK2 multiprotein complex after ligand recognition is a critical event required for the activation NF-κB/MAPK. Upon ligand-induced oligomerization of NOD1/2, RIPK2 is recruited via CARD-CARD interactions. Structural and mutagenesis studies of RIPK2 demonstrated that it forms long filamentous structures that are bound by NOD2 and that the polymerization of RIPK2 is important for NOD2 activation (68). The assembly and stability of NOD1/2-RIPK2 complexes requires the heme-regulated inhibitor (HIR), an eIF2a kinase, and the associated heat shock protein, HSPB8A, and the absence of these components results in impaired NF-κB-mediated production of inflammatory cytokines (85). As HIR promotes the solubility of NOD1 oligomers after activation, it has been proposed that the HIR/HSPB8 axis is necessary to promote the stability of large complexes including the NOD1 or NOD2 signalosome complex formation (85).

NOD1 and NOD2 have also been shown to induce Type I interferon (IFN) signaling, which plays an important role in antiviral immunity (86). For example, upon exposure to viral ssRNA, NOD2, but not NOD1, interacts with MAVS (mitochondrial antiviral signaling) via its CARD and NBD domain, resulting in activation of interferon regulatory factor-3 (IRF3) in a TRAF3-dependent manner and induction of IFNβ production (26, 87). Overexpression and knockdown of NOD2 also affected Type I IFN production in response to CMV, a dsRNA virus, which was also mediated, in part, by IRF3 (88). The addition of MDP can potentiate Type I IFN responses to CMV infection likely via induction of TBK1/IRF3/7 pathway (89). MDP alone has also been shown to induce Type I IFN responses (90), and this occurs via RIPK2-mediated activation of TBK1 and IRF5 in the context of *Mycobacterium tuberculosis* infection of macrophages (39).

In the case of NOD1, although not responsive to ssRNA like NOD2, NOD1 has been shown to interact with dsRNA and can augment *IFN-β* gene expression in certain cell types mediated by RIG-I or MDA5/MAVS (27, 28). NOD1 can also participate in Type I IFN signaling independently of MAVS. Stimulation of intestinal epithelial cells or hepatocytes with NOD1 ligand (i.e., ieDAP or TriDAP) induces IFN-β production that is dependent on RIPK2 (91, 92). In particular, stimulation of cells with NOD1 ligand results in RIP2 binding to TRAF3, which then leads to recruitment and activation of TANK-binding kinase 1 (TBK1), IκB Kinase e (IKKε), and IRF7 to induce IFN-β production and signaling via the ISGF3 pathway (92).

Activation of NOD1 and NOD2 can also lead to initiation of autophagy pathways. Specifically, stimulation of various cell types with NOD1 or NOD2 agonists induced autophagy (93) (94, 95). This phenomenon also occurred *in vivo* in peritoneal macrophages when mice were injected intraperitoneally with either NOD1 or NOD2 agonists. Induction of autophagy occurred independently of RIPK2 and NEMO, a component of NF-κB signaling; rather, NOD1 and NOD2 interacted with the autophagy regulator ATG16L1 at the plasma membrane, triggering autophagy, which can promote the sequestration of intracellular bacteria into autophagosomes to promote their subsequent clearance (93).

NOD 1 and NOD2 in adaptive immunity

Both NOD1 and NOD2 can participate in adaptive immunity. NOD1 and NOD2 agonists can act as adjuvants to enhance antibody production (96) and T cell responses. Immunization of NOD1-deficient mice with complete Freud's adjuvant which contain both TLR4 and NOD1 agonists, resulted in impaired antigen-specific antibody production and reduced numbers of IL17, IL-4, and IFN-γ producing T cells (97). This may, in part, be due to the ability of NOD1 ligand to induce stimulate DC responses alone and cooperatively with TLR4 agonists (97, 98). In addition, immunization of mice with NOD1 ligand and ovalbumin (OVA) results in an enhanced Th2 polarized antigen-specific response that required NOD1 function in non-hematopoietic cells, possibly as a result of the NOD1-mediated induction of chemokines, including the Th2-promoting factor, MCP (97) Similar to what occurs with NOD1 ligand, systemic release of MCP occurs after administration of MDP to mice, and immunization of mice with MDP and OVA results in a Th2 polarized antigen-specific T and B cell response that requires NOD2 function in non-hematopoietic cells and in $CD11c^+$ DCs (99, 100). In particular, both NOD1 and NOD2 promote the production of thymic stromal lymphopoietin protein (TSLP) production by non-hematopoietic cells and upregulate OX40 ligand (OX40L) surface expression on DCs that are important for Th2 immunity (100, 101). In addition, NOD2 is also capable of synergizing with TLR4 agonists to enhance the production of Th1 polarizing cytokines by DCs and prime Th1 cells as occurs with CFA immunization (99). Thus, while co-stimulation with NOD1 and/or NOD2 collaborate to a Th2 response, co-stimulation with TLR agonists can synergize to induce Th1, Th2 or Th17 immune responses (97-99). NOD1 and NOD2 may also promote specific T cell responses via other mechanisms that affect the priming function of DCs. For example, NOD2 stimulation induces autophagy in DCs, which is required from MHC class II (MHC-II) antigen presentation and antigen-specific CD4+ T cell responses (102). NOD2 can also affect miRNA expression in DCs that can affect the production of specific Th17 cell-polarizing cytokines (103). Injection of NOD1 or NOD2 ligands into mice also increases DC-mediated cross-presentation by enhancing antigen-presentation and costimulatory molecule expression on DCs (104). Altogether, these studies provide strong evidence for a role of NOD1 and NOD2 signaling in regulating adaptive immune responses.

NOD1 and NOD2 are also expressed in B and T cells, although level of expression is dependent on cellular location. There is some data to suggest that NOD1 and NOD2 ligands can enhance B and T cells after B-cell receptor (BCR) and T-cell receptor (TCR) engagement (105-107). Specifically, in human tonsillar B cells, NOD1 or NOD2 activation alone by treatment with their respective ligands was insufficient to trigger B cell activation or proliferation whereas the combination of NOD1 and NOD2 ligands and BCR activation via IgM or IgD stimulation resulted in enhanced cellular proliferation *in vitro*, which is further augmented by concomitant TLR stimulation (105). Stimulation of purified $CD3^+$ human tonsillar T cells with NOD1 or NOD2 ligands alone or after TCR activation with anti-CD3/CD28 did not induce cellular proliferation or T-cell cytokine production. Nonetheless, in mixed cultures of tonsillar mononuclear cells, enhanced cellular proliferation of T cells was observed with after anti-CD3/CD28 stimulation together with NOD1/NOD2 agonists although the effect was relatively weak (106). With purified murine splenic $CD3^+$ T cells, it was also demonstrated that NOD1 ligand can act as a costimulatory molecule to enhance IFN-γ production after stimulation with anti-CD3 alone; however, the possibility of a contributing factor by potential contaminating antigen-presenting cells was not ruled out (107). Regardless, as these studies were all performed *in vitro*, the physiologic significance of these findings remains to be determined *in vivo*.

Role of NOD1 and NOD2 in Host Defense

As sensors of peptidoglycan and regulators of key inflammatory pathways, such as NF-κB and MAPK, NOD1 and NOD2 play important roles in bacterial recognition and the activation of immune responses important for bacterial killing and clearance. Specifically, NOD1 and NOD2 promote host defense by inducing: 1) inflammatory cytokines that have bacterial killing activity, 2) chemokines that lead to the recruitment of neutrophils and macrophages to the sites of infection, 3) antimicrobial peptide production by epithelial cells, 4) Type I IFNs, 5) reactive oxygen species, 5) adaptive immune responses (53, 108), and 6) autophagy.

Largely through recognition of their respective peptidoglycan ligands, but also via peptidoglycan-independent mechanisms, NOD1 and/or NOD2 are capable of sensing *L. monocytogenes* (50, 96, 109), *Streptococcus pneumoniae* (110, 111), *Salmonella* (35,

47, 85, 108, 112, 113), *Mycobacterium tuberculosis* (94, 114), *Staphylococcus aureus* (115, 116), *E. coli* (117-119), *C. pneumoniae* (120), *Shigella flexeri,* and *Borrelia burgdorferi* (18, 36, 121-123).

Unlike NOD2 which is expressed primarily in myeloid cells, monocytes, macrophages and dendritic cells (3), NOD1 is ubiquitously expressed such as in epithelial cells (4). Thus, a common mechanism of NOD1-mediated host defense against bacterial pathogens is the induction of chemokines and antimicrobial peptides by epithelial cells that promote the recruitment of immune cells such as neutrophils and bacterial killing. *In vitro* stimulation of intestinal epithelial cells with NOD-1 synthetic ligands results in the production of CXCL1, CCL2 and IL-8, that are important for recruitment of neutrophils (124). Moreover, *in vivo* NOD1 stimulation can induce neutrophil recruitment into the peritoneal cavity after *i.p.* administration of NOD1 ligand; this response was abolished in NOD1-deficient mice (124). The ability of epithelial cells to respond to bacterial stimulation via NOD1 can be particularly important in the intestine where intestinal epithelial cells have low TLR expression and are less responsive to TLR stimulation with lipopolysaccharide (117, 125). In the case of *Clostridium difficile* which contains high NOD1-, but low NOD2-stimulatory activity, particularly in mesothelial cells, intraperitoneal injection resulted in significant neutrophil recruitment in the peritoneum that was NOD1-dependent, and NOD1-deficient mice had increased mortality to *C. difficile* colitis associated with impaired production of CXCL1, decreased neutrophil infiltration in the colon lamina propria and reduced bacterial clearance after oral gavage (126). Besides the intestine, NOD1 signaling in the gastric epithelium is important in host defense against *Helicobacter pylori* infection, which despite being a Gram-negative bacteria, does not activate TLR4 signaling in the gastric mucosa (53, 127). Peptidoglycan-containing *H. pylori* can activate both NF-κb and MAPK pathways that are NOD1-dependent (53, 128). NOD1-deficient mice had increased susceptibility to *H. pylori* infection resulting in reduced production of the chemokine MIP-2 and increased bacterial loads (53). Other mechanisms besides chemokine production likely contribute to clearance of *H. pylori* including production of antimicrobial peptides such as β-defensins (129, 130), induction of autophagy-induced inflammatory responses (40, 131) and synthesis of Type I IFNs by epithelial cells (92). NOD1 also promotes adaptive immune responses to *H. pylori* as

NOD1-deficient mice exhibit reduced IgG levels in response to *H.pylori* OMVs (62), which may be related to a role for NOD1 in regulating release of processed IL-33 (132), a key driver of Th2 immune responses, and NOD1-dependent Type I IFN production can lead to enhanced Th1 chemokines secretion (92). Thus, NOD1 engages multiple pathways to mediate host resistance to pathogens.

NOD2 has a more restrictive expression pattern than NOD1 with expression primarily in monocytes and macrophages. Besides the production of inflammatory cytokines, like NOD1, NOD2 can also upregulate chemokine production via NF-κB and MAPK pathways to recruit immune cells important for eradication of bacterial pathogens (69, 133). For example, *in vivo*, NOD2 promotes the CC-chemokine ligand 2 (CCL2)-CCR2-dependent recruitment of Ly6C^{high} monocytes in the intestine and clearance of *Citrobacter rodentium*-infected mice (134). *S. pneumoniae* also induces CCL2 via NOD2, leading to the recruitment of inflammatory macrophages that play a role in bacterial clearance in the lung (111).

An important function of NOD1 and NOD2 is to act cooperatively with Toll-like receptors (TLRs) to enhance immune responses against pathogenic infection as well as promote immune responses after tolerization by TLR ligands. For example, bone marrow derived macrophages (BMDMs) or mice stimulated with the TLR4 ligand, lipopolysaccharide (LPS), in combination with MDP, resulted in enhanced activation of NF-κB and increased production of IL-6 and TNF-α. In addition, BMDMs made insensitive or tolerant to TLRs by previous exposure to TLR ligands, such as LPS, exhibited enhanced response to NOD1 and NOD2 stimulation (135). The significance of this was demonstrated *in vivo* in which *Nod1*$^{-/-}$*Nod2*$^{-/-}$ mice previously infected with *E. coli* had decreased bacterial clearance and mouse survival after subsequent infection with *Listeria monocytogenes*, which contains both NOD1 and NOD2 ligands. Similarly, although activation of NF-κB and MAPK and production of inflammatory cytokines in BMDMs to *M. tuberculosis* was not dependent on NOD1, immune responses to *M. tuberculosis* was partly mediated by NOD1 signaling after tolerization of TLRs by LPS pre-treatment (136). These results demonstrate the importance of NOD1 and NOD2 signaling in host defense especially in the setting of secondary bacterial infections when TLR responses are impaired as a result of tolerization (135).

NOD1 and NOD2 signaling are also important for antiviral immunity via different mechanisms. *Nod2*$^{-/-}$ mice have been known to have increased susceptibility against a variety of viral pathogens (26, 87, 137). NOD2 is capable of interacting with ssRNA, and infection of NOD2-expressing cells *in vitro* or mice *in vivo* with VSV, RSV and influenza leads to upregulation of Type I IFNs that is NOD2-dependent (26). Infection of *Nod2*$^{-/-}$ mice with RSV develop worse lung pathology compared to that of wildtype mice (26). *Nod2*$^{-/-}$ mice also exhibit reduced survival and clearance of influenza virus after infection, which is associated with impaired DC responses and reduced priming of $CD8^+$ T cells to influenza (87). NOD2 is also capable of limiting influenza-induced lung pathology, by the induction of autophagy, and more specifically mitophagy, resulting in decreased mitochondrial damage and suppression of inflammasome activation that can contribute to immunopathology and morbidity (138). Although NOD1 mice can similarly respond to specific viruses *in vitro*, whether direct recognition of viruses of NOD1 affects disease outcomes remains to be determined.

Host antiviral responses can also be potentiated by stimulation of NOD1 and NOD2 by their respective PGN ligand. For example, MDP treatment of mice infected with influenza resulted in the induction of Type I IFNs as well as CCL2 resulting in increased recruitment of inflammatory monocytes, reduced viral loads, improved lung pathology and decreased mortality that was NOD2-dependent (139). Stimulation of NOD2 by MDP also inhibited cytomegalovirus (CMV) replication *in vitro* and was associated with the synergistic induction of Type I IFNs by CMV and MDP (89). Liver sinusoidal endothelial cells stimulated with the NOD1 ligand diaminopelic acid (DAP) resulted in maturation of liver sinusoidal endothelial cells and increased T cell responses *in vitro* (140). Consequently, administration of DAP *in vivo* resulted in increased Hepatitis B virus (HBV)-specific T cell responses *in vivo* and replication of HBV (140). Mice pre-treated with the NOD1 ligand ie-DAP also exhibited reduced CMV load after infection (91), and the combination of ie-DAP and MDP enhanced inhibition of CMV replicative activity which required Type I IFNs (91). These results suggest that exposure to bacteria and stimulation of NOD1 and NOD2 can augment antiviral immunity. Conversely, it has also been demonstrated that Type I IFNs can induce expression of NOD1 and NOD2 in BMDMs resulting in enhanced NF-κB/MAPK activation and inflammatory cytokine production.

Consistently, infection of bone marrow macrophages by murine norovirus (MNV) resulted in increased NOD1- and NOD2-dependent activation of NF-κB/MAPK activation and production of TNF-α and IL-6. Although this phenomenon may have been designed to enhance immune responses against a secondary bacterial infection, *in vivo* infection by bacteria such as *E. coli* after MNV infection resulted in increased inflammation and mortality in mice, which is reminiscent of the negative consequences of secondary bacterial infections in humans due to an overexuberant inflammatory response (137).

A role for NOD1 and NOD2 in fungal and parasitic infections has been less well-studied. Addition of *Aspergillus fumigatus* to immortalized human corneal epithelial cells results in increased expression of NOD1 and RIPK2 (141). However, although knockdown of *Nod1* results in reduced production of inflammatory cytokines in response to *Aspergillus* (141), *in vivo* infection in NOD1-deficient mice was associated with increased survival, reduced inflammation, and improved clearance, suggesting that NOD1 signaling, in fact, suppresses immune responses against *Aspergillus*, possibly due to NOD1-mediated reduction in Dectin-1, a C-type lectin pattern recognition receptor, which is important for *Aspergillus* killing (142). Similarly, NOD2-deficient mice were also protected against invasive *Aspergillus* and negatively regulated Dectin-1 expression (143). NOD2 also has negative regulatory role in the immune response against chitin, a polysaccharide of the fungal cell wall (144). Fungal chitin, which forms a component of PGN, induces the release of the anti-inflammatory cytokine IL-10 by BMDMs via NOD2 and TLR9 whereas the production of TNF-α was dependent on TLR2 and Dectin-1 (144). Intracellular delivery of chitin was required and mediated by the mannose receptor, a C-type lectin PRR, which is capable of actin remodeling and mediating both phagocytosis and endocytosis (145). Importantly, the chitin-induced anti-inflammatory response may play a critical role during the late phase of infection to allow resolution of inflammation (144).

Both NOD1 and NOD2 have also been shown to sense certain parasitic infections. In particular, NOD1-deficient mice were more susceptible to *Trypanosoma cruzi* infection compared to that of wildtype (WT) mice. NOD2 was also shown to be important for resistance against *Toxoplasma gondii* infection by inducing Th1 responses (146). NOD2-deficiency also resulted in increased susceptibility to *T. gondii*-induced ileitis and cerebral

inflammation after oral infection (147). The impairment in mounting Th1 responses in NOD2-deficient mice against *T. gondii* was not due to a defect in DC priming, but T cell-intrinsic role for NOD2 in regulating IL-2-deficient mice via non-canonical NF-κB signaling (146). However, a separate study was unable to demonstrate a protective role for NOD2 against *T. gondii* infection or a T-cell intrinsic function (148), which was posited to be due either insufficient backcrossing of NOD2-deficient mice used in the previous study or differences in the gut microbiota in mice in different mouse facilities. NOD2 was also suggested to recognize the malarial pigment hemozoin (149), and *Nod1*$^{-/-}$*Nod2*$^{-/-}$ mice exhibited reduced inflammatory cytokine production in response to *Plasmodium berghei*; however, there was no differences in survival or susceptibility to cerebral malaria (150). Thus, how NOD1 and NOD2 sense parasites and whether they play a role in disease susceptibility remains to be fully elucidated.

NOD1 and NOD2 in intestinal homeostasis

Through their regulation of pro-inflammatory cytokines/chemokines, anti-microbial peptides, adaptive immunity, and autophagy, NOD1 and NOD2 can promote intestinal homeostasis by enhancing epithelial barrier function (151, 152) and resistance to pathogen invasion that can lead to infectious colitis. Activation of NOD1 and NOD2 by *Salmonella* infection in the intestine increases clearance and limits the severity of *Salmonella* colitis in mice (108). NOD2-mediated regulation of CCL2 secretion and Th17 responses protects mice against *Citrobacter rodentium*-induced colitis (108, 134). NOD1 signaling promotes neutrophil recruitment via the induction of chemokines which is important for reducing the severity of *Clostridium difficile* colitis (126). Intestinal bacterial infections by *L. monocytogenes, S. flexeri*, and *Helicobacter hepaticus* are also ameliorated as a result of NOD1 and NOD2 activation via the production of pro-inflammatory mediators, anti-microbial peptides, and/or bacterial autophagy (93, 96, 153).

NOD1 and NOD2 also play other important functions to help maintain intestinal homeostasis. NOD2 is constitutively expressed in LGR5+ intestinal stem cells (ISCs) for example. Activation of NOD2 is associated with increased intestinal organoid-forming efficiency and *in vivo* treatment of mice with MDP improved intestinal epithelial regeneration and healing after doxorubicin-induced injury associated with greater stem

cell activity (154) although the precise mechanism for this protection remains to be determined. NOD1 signaling in non-hematopoietic cells regulates the development of isolated lymphoid follicles (ILFs) in the intestine, and treatment of germfree mice with a NOD1, but not NOD2 agonist, resulted in increased ILF formation, particularly in the ileum via NOD1-dependent induction of CCL20 and beta-defensin3, both of which promote the formation of ILFs (155). How NOD1-mediated induction of ILFs affects susceptibility to intestinal disease is not clear; however, the ileal biofilm of NOD1-deficient mice contained a greater bacterial load than that of WT mice as well as an expansion of Gram-negative bacteria, including *Bacteroides* and *Enterobacteraceae*. As ILFs are important sites of IgA induction (156), it is possible that an effect on IgA production may have contributed to the altered microbiome composition as IgA-deficient mice exhibit similar changes (155, 157). Whether these effects on the gut microbiota lead to disease susceptibility remains to be determined.

Significant evidence pointing to the importance of NOD2 in maintaining intestinal homeostasis comes from studies pointing to a link between NOD2 dysfunction and inflammatory bowel disease (IBD). IBD is a chronic, relapsing-remitting inflammatory disorder involving the gastrointestinal tract. There are two forms of IBD, ulcerative colitis and Crohn's disease, and they are distinguished from each other based on clinical characteristics and histopathologic characteristics. Ulcerative colitis affects just the colon, particularly the rectum, and only the surface mucosal layer. In contrast, Crohn's disease can affect any part of the GI tract, although occurs commonly in the ileum, and inflammation is transmural and not necessarily contiguous ("skip" lesions). Although the pathogenesis of IBD is still not fully understood, the prevailing model is that combined genetic and environment factors lead to disruption of the integrity of the epithelial barrier, pathologic changes in the composition of the gut microbiome, referred to as dysbiosis, and aberrant immune responses to the gut microbiota (158). NOD2 has been identified as a major susceptibility gene for Crohn's disease, and multiple polymorphisms, including loss-of-function mutations have been associated with increased risk of developing Crohn's disease (159, 160) (*Figure 5A, Table 2*). Individuals harboring NOD2 homozygous or compound heterozygous for NOD2 variants exhibit 20- to 40-fold

increased risk of developing CD (161). Although conferring CD risk, the majority of individuals carrying NOD2-associated polymorphisms do not develop disease.

How NOD2 regulates susceptibility to developing IBD has been the focus of significant investigation, but several mechanisms have been proposed (*Figure 5B*). The most common CD-associated Nod2 variant is an L1007fs frameshift insertion at nucleotide 3020 (3020insC) in the LRR domain that generates a truncated protein (3, 159, 162). Monocytes from homozygous individuals showed impaired cytokine response after MDP stimulation (163), which was similarly observed in mice BMDMs carrying the *Nod2* mutation homologous to the human L1007fsinsC ($Nod2^{2939iCstop}$) (164) or in cells transfected with mutant NOD2 in response to MDP (121, 162). This mutant version of NOD2 does not localize to the plasma membrane (43, 165), and consequently does not recruit ATG16L, thereby preventing the formation of autophagosomes and impairing bacterial clearance (93). The important of the NOD2-ATG16L pathway is further supported by the observation that individuals homozygous for the single nucleotide polymorphism rs2241880 in the ATG16L gene are associated with increased risk for Crohn's disease (166, 167), and cells from these individuals have defective autophagy after MDP stimulation (93). Crohn's disease variants in NOD2 have also been shown to have defective S-palmitoylation, which is required for plasma membrane association and optimal bacterial sensing (47). DCs isolated from individual harboring either the 1007fsinsC Nod2 or the Crohn's variant ATG16L T300A exhibited defective autophagy, MHC II processing, antigen-specific $CD4^+$ T cell responses against bacteria, which can reduce mucosal immunity (102). In addition, DCs harboring Crohn's disease *Nod2* variants have impaired levels of mir-29 as a result of impaired NOD2 signaling, resulting in enhanced release of IL-23 and IL12p40 which can contribute to disease (103). It has also been shown that BMDMs from a different mouse strain that harbors the same homologous 1007fsinsC ($Nod2^{2939ic}$) allele exhibited increased NF-κB activation and heightened cytokine responses consistent with the observation that Crohn's disease patients have increased production of inflammatory cytokines (168). $Nod2^{2939ic}$ mice also exhibited increased susceptibility to chemically-induced colitis with dextran sulfate sodium (DSS) (168), a commonly used mouse model that has features that recapitulate that of human IBD. The difference in BMDM responses to MDP between the two different

mouse strains is not clear, but it was postulated that this discrepancy may be due to the addition of amino acids after the frameshift mutation in *Nod2*^{2939ic} mice that are not present in the wildtype NOD2 protein or in the truncated Crohn's disease-related mutant protein that is present in *Nod2*^{2939ic} mice (164).

Mutations in NOD2 have been linked to disease localization to the ileum (169, 170). Notably, Paneth cells are located in the crypts of the small intestine, and abnormalities in Paneth cells have been noted in Crohn's disease patients (171). Paneth cells are important for the production of antimicrobial peptides (AMPs). Decreased alpha-defensin levels have been observed in Crohn's disease patients with *Nod2* mutations (172). In mice, NOD2 deficiency is associated with decrease in AMP production, specifically cryptdins, resulting in impaired gut barrier function and increased susceptibility to bacterial infection (96). Under steady-state conditions, AMPs, such as lysozyme, are synthesized in the ER and transported to the Golgi, where they are packed into immature secretory dense core vesicles (DCV) in Paneth cells. NOD2 stimulation allows the proper lysozyme sorting during DCV maturation, and in the absence of NOD2, lysozyme degradation and depletion are observed, which can contribute to decrease bacterial clearance (173, 174). Other abnormalities in the small intestinal epithelium have been observed with NOD2 deficiency, including decreased MUC2 expression and reduced goblet cell numbers, which may lead to reduced mucus production and barrier function (175).

Mice that lack NOD2 or harbor the L007framshift mutation do not develop spontaneous colitis (96, 168), consistent with the fact that most individuals who have this mutation do not develop disease, suggesting that other factors, including environmental, likely contribute to disease pathogenesis. There is growing evidence that pathologic changes in the gut microbiome, or dysbiosis, can directly contribute to IBD. In particular, the microbiome of IBD patients typically exhibit a loss of microbial diversity, expansion of *Enterobacteriaceae* and loss of *Faecalibacterium prausnitzii* (176). NOD2 deficiency in mice have been associated with changes in microbiome composition, which may be related to defects in mucosal barrier function and microbial sensing. NOD2 knockout mice exhibited increased bacterial loads in the ileum as well as higher abundance of Bacteroidetes and decreased levels of Firmicutes in the intestine, similar to what has

been observed in Crohn's disease patients harboring SNP13 variants, suggesting a role for NOD2 in regulating gut microbiome composition, which in turn can affect susceptibility to colitis (177-179). Consistently, fecal transplantation of stool from NOD2-deficient mice into germfree mice resulted in increased susceptibility to DSS-induced colitis compared to that of germfree mice harboring microbiota from WT mice(180). In a separate study, NOD2 deficient mice were found to exhibit an expansion of *Bacteroides vulgatus,* which can promote piroxicam-induced small intestinal inflammation, resulting in further changes in the microbiota, including increases in Proteobacteria and decreases in *Bacteroidetes* and *Clostridiales* (175). NOD2 polymorphisms may also promote disease through defects in sensing protective signals from the microbiome that are important for gut homeostasis. For example, it has been shown that OMVs from the commensal *Bacteroides fragilis* OMVs can trigger the autophagy pathway in DCs, which in turn, induces regulatory T cells in the gut associated with protection against chemically-induced colitis that is NOD2-dependent (181). Thus, the loss of NOD2 function may lead to impairment of bacterial recognition and clearance resulting in aberrant inflammation through other inflammatory pathways, including TLRs. As NOD2 has also been shown to directly negatively regulate TLR signaling, defective NOD2 signaling may further enhance immune responses to dysbiosis and increase colitis susceptibility (182-184).

As mentioned above, multiple mouse studies have shown a protective effect of NOD2 against multiple models of intestinal inflammation (175, 180, 182), and treatment of mice with MDP resulted in protection from both DSS- and trinitrobenzene sulfonic acid (TNBS)-induced colitis (185). However, there have also been studies demonstrating no effect of NOD2 in ameliorating DSS-induced colitis (186). In addition, in the SAMP1Yit/Fc (SAMP) mouse model of spontaneous ileitis, NOD2 deletion improved intestinal inflammation in the presence and absence of DSS and was associated with reduced Th2 responses (187). No significant differences in overall microbiome composition were observed at least on a community level between SAMP1Yit/Fc and wildtype mice (187). As the gut microbiome can drive host immune responses in the gut, the discrepancy in findings from *in vivo* mouse studies are likely dependent not only on the strain of mice, but also on microbiome differences between mouse facilities. It is also important to note that it is unclear whether previous studies used littermate WT and *Nod2*$^{-/-}$ mice, and

therefore, it is also possible that any observed effects in colitis susceptibility were related to differences in the gut microbiota from vertical transmission not related to NOD2 deficiency. Future studies using littermate mice or conventionalized GF WT and NOD2-deficient mice will be necessary to further delineate the effects of NOD2 on microbiome and colitis susceptibility.

NOD1 have also been associated with IBD although to a much lesser extent with gene polymorphisms associated with IBD risk identified in only specific populations (188-191). Although there is strong evidence that NOD1 can limit infectious colitis in mice by specific pathogens, such as *C. difficile*; there are limited studies evaluating the role of NOD1 in colitis. In a mouse model of colitis-associated tumorigenesis which combines the injection of the experimental carcinogen, azoxymethane (AOM), with multiple rounds of DSS to induce chronic, relapsing colitis, NOD1-deficient mice were found to have increased severity off AOM/DSS-induced colonic inflammation compared to WT mice, which was associated with reduced epithelial barrier integrity and increased intestinal epithelial permeability (192). Other studies evaluating *Nod1*$^{-/-}$*Nod2*$^{-/-}$ mice also demonstrated increased chemically-induced colitis severity in these mice compared to that of wildtype mice (193). Additional studies using littermate or germfree *Nod1*$^{-/-}$ mice to evaluate NOD1-specific effects on colitis susceptibility and any microbiome-dependent contributions will need to be performed to further understand NOD1 function in intestinal homeostasis.

NOD1 and NOD2 in systemic inflammatory disease.

Type 1 diabetes

Predisposing genetic background and environmental insults can give rise to autoimmune disorders, such as Type 1 diabetes (T1D) (194-196). T1D is characterized by chronic inflammation involving the pancreatic islets of Langerhans and irreversible elimination of pancreatic beta-cells mediated by autoreactive T cells leading to insulin deficiency and hyperglycemia. Besides the well-known involvement of adaptive immunity in the pathogenesis T1D, there are studies to suggest a potential role for innate immunity, including NOD1 and NOD2, although this has been less well-studied. Environmental factors that affect gut permeability and alterations in the composition of the intestinal

microbiota all can affect NOD1 and NOD2 signaling to trigger inflammation and promote T1D (196-198). For example, in a mouse model of streptozocin-induced T1D mice exhibited changes in the gut microbiota and translocation of bacteria into pancreatic lymph nodes, which preceded the development of T1D (199) and led to upregulation of NOD2-dependent inflammatory responses in macrophages and DCs. Treatment of mice with MDP in this model promoted the development of hyperglycemia (200). On the other hand, NOD2-deficient non-obese diabetic (NOD) mice had increased susceptibility to developing T1D although this was microbiota-dependent. Clearly, additional studies are necessary to clarify the importance of NOD1 and NOD2 in T1D.

Type 2 diabetes

There is a compelling evidence that dysregulated NOD1 and NOD2 signaling can also affect risk for developing type 2 diabetes (T2D), a chronic inflammatory condition that is typically associated with insulin resistance and obesity, although studies are conflicting in terms of their protective versus detrimental roles (201-204). Endotoxemia due to changes in intestinal permeability and translocation of PAMPs to the circulation is observed in mouse models of T2D, which can give rise to inflammation and insulin resistance (205). Individuals with metabolic syndrome, who are at high risk for developing insulin resistance and T2D, exhibit high expression and activity of NOD1 (206). Increased intestinal permeability and translocation of bacteria systemically in mice on a high fat diet (HFD) led to increased circulating levels of NOD1 agonists, and deficiency in NOD1 resulted in a protective effect with reduced insulin resistance, glucose intolerance, and HFD-induced inflammation (204, 207). Administration of NOD1-activating muropeptides also worsened glucose tolerance (207). ER stress is also highly associated with insulin resistance since mice deficient for XBP-1, a regulator of the unfolded protein response, develop diet-induced insulin resistance (208). Saturated fatty acids, which can be sensed by NOD1, also can activate the unfolded protein response and induce ER stress, via ER calcium depletion, to induce inflammation and insulin resistance (209). Therefore, it is possible NOD1 is linked to ER stress that occurs during the development of HFD-induced obesity that potentiates systemic inflammation and predisposes to T2D (210).

On the other hand, NOD2-deficient mice developed increased insulin resistance and adipose tissue compared to WT mice on HFD (203). In a separate study, *Nod2*$^{-/-}$ mice on HFD exhibited increased intestinal bacterial translocation into metabolic tissues (*e.g*. hepatic and adipose) ,which was associated with inflammation and insulin resistance due to the microbiota changes in NOD2-deficient mice (211). Accordingly, mice injected with the NOD2 ligand MDP showed decreased insulin resistance in obese mice (202). Altogether, these results suggest divergent roles of NOD1 and NOD2 in T2D development; however, the mechanisms involved still remain unclear.

Atherosclerosis

Atherosclerosis is a vascular disease characterized by atheromatous plaques in the intima as well as systemic inflammation. The cell death of vascular smooth muscle cells (VSMCs) in the necrotic core of advanced plaques may imperil the atherosclerotic lesion and thin the protective fibrous cap due to decreased collagen production, leading to cellular debris accumulation and intimal inflammation, causing plaque vulnerability which can lead to myocardial infarction (212, 213). ER stress has been observed in VSMCs in the atherosclerotic plaques after perturbation (214, 215). NOD2 is expressed in VSMCs (216), and plays protective effects in a vascular injury model of neointima hyperplasia by promoting VSMCs proliferation, migration, and neointimal formation after vascular injury (217). NOD2-deficient VSMCs also enhance ER stress-induced cell death (218). Finally, NOD2 ablation promoted disruption of atherosclerotic lesions in *ApoE*$^{-/-}$ mice under an atherogenic diet (218). These data demonstrated a possible protective role of NOD2 during ER stress-induced VSMC death in processes related to plaque necrosis and progression of atherosclerotic lesions.

NOD1 and NOD2 in autoinflammatory diseases

Blau syndrome/early onset sarcoidosis

The autoinflammatory granulomatous diseases Blau syndrome (BS) and early-onset sarcoidosis (EOS) are associated with excessive inflammation and granuloma formation. Both diseases are phenotypically similar, characterized by arthritis, skin rash, and uveitis, and histologically by the occurrence of noncaseating epithelioid granulomas

that typically present between 3 and 4 years of age (219, 220). BS and EOS share a common genetic etiology, namely, gain-of-function single nucleotide polymorphisms (SNPs) in the NOD2 gene leading to an auto-activation of NOD2-mediated NF-κB signaling (221-224). NOD2-related BS/EOS polymorphisms were shown to cluster into the ATP/Mg^{2+}-binding site and helical domain 1 (HD1) in the NOD2 NOD domain, which may dysregulate ATP hydrolysis and NOD2 autoinhibition, respectively (225) (*Table 2*). Complementary mutations in NOD1, however, do not mirror the NOD2 phenotype, which suggest that NOD1 and NOD2 are activated and regulated by distinct methods (222). The mechanism by which NOD2 mutations participate in granulomatous inflammation still remains to be clarified.

While most of NOD2 mutations associated with CD susceptibility are located between the NBD and LRR domains (*Figure 5*), BS mutations primarily affect the NBD domain with the missense mutation at position 334 (either p.R334Q or p.R334W) found to be the most common and severe (223, 226-230). These mutations enhance the self-oligomerization of NOD2 increasing its activity, even in the absence of MDP. Consistently, it was reported that IL-1β, IL-6, and TNFα levels in the plasma of BS patients were significantly higher than those of healthy controls (227). Overexpression of disease-causing mutations in cell lines was also associated with enhanced NF-κB activation (225, 231).

SNPs in the NOD2 associated with loss-of-function have also been described (222, 232)(Table I). However, there is only weak evidence pointing to NOD2-loss of function in BS development. Notably, *in vitro* NF-κB activity related to the overexpression of BS NOD2 mutations was not considered a good predictor of BS/EOS severity although the number of patients evaluated was small (233). Also, mice carrying the BS/EOS p.Arg314Gln mutation do not develop spontaneous disease, but instead show reduced circulating inflammatory cytokines after MDP stimulation, accompanied by decreased macrophage activity and RIPK2, MAPK and NF-κB activation in these cells (234). Thus, BS/EOS result primarily from the over-activation of NOD2, rather than a loss-of-function of NOD2 as observed in Crohn's disease.

Sarcoidosis

In contrast to BS/EOS, the primary site of sarcoidosis granuloma formation is in the lung. Although BS and EOS were reported to share identical NOD2 mutations, no association has been reported between NOD2 and sarcoidosis (235). Instead, NOD1 polymorphisms were found in patients with sarcoidosis (236) (*Table 2*).The *Nod1* 796-allele was shown to diminish NF-κB activation in response to intracellular *Cutibacterium acnes*, a bacterium that has been isolated from sarcoid lesions and has a slightly higher odds of being detected in sarcoidosis patients than in healthy individuals, (236-238). Regardless, sarcoidosis is considered a non-infectious granulomatous disease, and its etiology remains unknown. NOD1 activation can also contribute to heightened inflammation in sarcoidosis. Sustained activation of p38 MAPK in response to NOD1 agonist and a lack of negative feedback loop via mitogen-activated protein kinase (MKP1) have also been shown to lead to inflammation in chronic sarcoidosis. Consistently, overexpression of MKP1 or chemical inhibition of p38 activation abrogated NOD1-mediated Th1 cytokine production (239). Additional studies evaluating bacterial contributions and the function of NOD1 in the pathogenesis of sarcoid are still needed.

Asthma

NOD1/NOD2/RIPK2 signaling has also been implicated in the development of Th2 immune responses and might play a critical role in the pathology of Th2-mediated conditions such as asthma and atopy. NOD2 is significantly upregulated in asthma patient tissues, and its overexpression in human airway smooth muscle cells (HASMC) promoted proliferation and pro-inflammatory cytokine release in a manner dependent of TSLP (240). Mice immunized with OVA and NOD1 or NOD2 agonist exhibit Th2-skewed T cell responses (99, 100). In a mouse model of house dust mites (HDM)-induced asthma, ablation of RIPK2 was sufficient to diminish Th2 inflammatory responses and pathology in the lung, indicating that NOD1 and NOD2 signaling can contribute to allergic airway inflammation (241). It was also demonstrated that NOD-1-mediated exacerbation of allergic asthma require the DC-derived pro-Th2 chemokine CCL17 (242). These data suggest that NOD1 and NOD2 activation may influence the development of asthma. Importantly, polymorphisms in *Nod1, Nod2,* and *Ripk2* genes were associated with allergy and asthma (243-246), and a genome-wide association study point to a link between

NOD1 polymorphisms, asthma, and high levels of serum IgE (247) (*Table 2*). How these polymorphisms affect NOD1 function and disease pathogenesis remains to be elucidated.

CNS inflammatory disease

Both NOD1 and NOD2 have been shown to play a role in the development of autoinflammatory disease involving the CNS, specifically multiple sclerosis, an inflammatory demyelinating disease, via recognition of PGN (248). PGN is present within antigen-presenting cells (APCs) in the CNS of multiple sclerosis (MS) patients (249) as well as in CNS lesions in animal models of MS (250, 251), suggesting that the presence of PGN, possibly as a result of prior bacterial infection may contribute to the pathogenesis of multiple sclerosis (252). In experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis, PGN can induce disease by stimulating dendritic cells to promote autoimmunity (250) and also able to induce EAE in mice (250, 253). Importantly, recognition of PGN via NOD1 and NOD2 promoted EAE progression, and $NOD1^{-/-}$, $NOD2^{-/-}$ and $RIPK2^{-/-}$ mice were resistant to EAE progression reduced numbers of T cells and activated DCs in the CNS of knockout mice (248). APCs isolated from $RIPK2^{-/-}$ mice with EAE were defective in their ability to drive Th17 effector cell differentiation whereas RIPK2 function in T cells was not required for disease progression. Altogether, these findings suggest that activation of NOD1 and NOD2 signaling by PGN is required for the activation of CNS-infiltrating DCs, resulting in reactivation of myelin-specific T cells, which, in turn, mediate pathogenesis of EAE. The clinical implication of these findings is significant as they point to a potential therapeutic target in the treatment of multiple sclerosis; however, it remains to be determined the significance of NOD1 and NOD2 signaling in the pathogenesis of multiple sclerosis as there have been no polymorphisms in NOD1 or NOD2 identified that are associated with human disease (254).

Conclusion

It has become clear that NOD1 and NOD2, as sensors of peptidoglycan and cellular stress and activators of multiple signaling pathways involved in immunity and tissue repair, are key players in the maintenance of health and resistance to infectious

and inflammatory diseases. Despite significant advances that have been made using mice models and *in vitro* systems, questions still remain regarding the precise mechanism by which NOD1 and NOD2 signaling contribute to disease pathogenesis. In particular, carefully controlled studies that dissect and separate potential interactions between NOD1/NOD2 and the gut microbiota and how this crosstalk affects the development, maintenance, regulation, and activation of the innate and adaptive immune system in health and in disease are still needed. Epigenetic mechanisms of NOD1 and NOD2 molecular signaling and the identity of NOD1 and NOD2 interacting partners that are involved in recognition of upstream activators also remain to be fully elucidated. Such studies could lead to preventive and/or therapeutic options for health maintenance or diseases related to dysfunction of NOD1/NOD2 signaling pathways.

Conflict of interest: None

Acknowledgments

We would like to thank Dr. Patricia A. Assis for assisting in the conceptualization and preparation of Figures. Work in the laboratory of G.Y.C is supported by NIH grant R01DK122812, USDA grant NIFA 2018-67017-27520 and DOD grant CA170922 to G.Y.C.

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Figures Legends

Figure 1. Schematic representation of NOD-like receptors 1 and 2. NOD1 consists of one N-terminal caspase activation and recruitment domain (CARD) and NOD2 has two in tandem. In both receptors, the domain CARD is followed by a core nucleotide-binding domain (NBD) and a C-terminal leucine-rich repeat domain (LRR). In the absence of ligand, NOD1 and NOD2 are in an inactive monomeric form, maintained by the binding of LRR domain into NBD and stabilized by chaperone proteins, such as HSP70 or HSP90. Upon recognition of PGN (peptidoglycan) ligands a conformational change occurs, resulting in homo-oligomerization of two NOD molecules that once activated trigger inflammatory signaling pathways.

Figure 2. Basic structure of peptydoglycan (PGN) motifs recognized by NOD1 and NOD2. Lys-PGN are present in Gram-positive bacteria, whereas DAP-PGN constitute the cell wall of gram-negative bacteria. Abbreviations: D-Ala: D-Alanine; D-Glu: D-Glutamic acid; GlcNAc: N-acetylglucosamine; L-Ala: L-Alanine; L-Lys: L-Lysine; mDAP: meso-diaminopimelic acid; MurNAc: N-acetylmuramic acid; iE-DAP: γ -D-glutamylmeso-diaminopimelic acid ; M-Tetra-DAP: MurNAc-L-Ala-D-Glu-mDAP-D-Ala; M-TriDAP: MurNAc-L-Ala-D-Glu-mDAP.

Figure 3. Mechanisms of PGN entrance into host cells to trigger NOD signaling. Host cells can internalize PGN, such as, MDP through different mechanisms: **(A)** phagocytosis of bacteria can release PGN in the cytoplasm after degradation of bacteria on the phagosome. Some pathogenic bacteria can evade the phagosome and replicate in the host cell, thus releasing PGN in the cytoplasm; **(B)** Extracellular PGN fragments can enter the host cell through endocytosis and transported to the cytosol through

SLC15A3/4, lysosomal membrane transporters. Alternatively, the dipeptide transporter hPepT1, expressed in the intestine, can also carrier free PGN fragments toward the host cell; **(C)** Some bacteria can delivery PGN into the host cell cytoplasm through its secretion system; **(D)** Uptake of outer membrane vesicles (OMVs) released by Gram-negative bacteria facilitates internalization of PGN.

Figure 4. NOD1 and NOD2 signaling pathways. Sensing of iE-DAP and/or MDP occurs via the LRR domains by NOD1 and NOD2, respectively. Once activated, NOD receptors recruit the kinases RIP2 which can interact with LUBAC and the kinase complex TAK1:TAB. This complex of proteins can activate two different pathways: 1) Activation of the IKK complex (NEMO: IKKα: IKKβ) which in turn activates NF-κB nuclear translocation; 2) activation of MAP kinases (e.g., p38, ERK and JNK) will activate the transcription factor AP1. Both NF-κB and MAPK pathways will induce gene expression of pro-inflammatory cytokines and mediators, including antimicrobial peptides (AMP). Alternatively, ER stress triggers the Unfolded Protein Response (UPR) caused by accumulation of unfolded or misfolded proteins and bacterial infection. ER stress activates IRE1α (inositol-requiring enzyme 1α) which recruits TRAF2, NOD receptors and RIPK2 to the ER membrane and initiating inflammatory response through NF-κB signaling; however, the exact mechanism of NF-κB signaling activation by ER stress is still unclear. A cytosolic UPR (cUPR) has been described that is required for NOD1 and NOD2 complex formation and activation of NF-κB. Upon PGN recognition, the heat shock protein HSPBB8 is released from the complex with HRI and binds NOD1 or NOD2 allowing the folding and release of these receptors from endomembranes. This pathway is associated with NF-κB activation, although the mechanism is not totally known. At the bacterial entry site on the plasma membrane, NOD2 can recruit the autophagy protein ATG16L1, leading to elimination of intracellular pathogens. NOD1 and NOD2 signaling can also induce type I Interferons expression. NOD2-RIPK2 activates of TRAF3 which recruits TBK1 and induces the IFN-β transcription factor IRF7. In addition, NOD2 is also activated by sensing of virus-derived single strand RNA (ssRNA). Binding of NOD2/TRAF3 to mitochondrial antiviral signaling (MAVS) induces activation of IRF3 which induces IFN-β gene expression.

Figure 5. NOD2 Polymorphisms in IBD. (A) Mutations in the NBD domain are associated with Blau Syndrome and single nucleotide polymorphisms in the LRR domain are associated to Crohn's Disease. **(B)** NOD2 mutations results in poor sensing of MDP fragments impairing NF-κB activation and decrease production of antimicrobial peptides (AMPs) by Paneth cells. NOD2 variants also fails to recruit ATG16L1 resulting in impaired autophagy by epithelial cells. Dysregulation of these mechanisms leads to reduced bacterial clearance and loss of mucosal barrier function. NOD2 is also important for maintenance of the goblet cell number and mucus secretion. In addition, loss of commensal bacteria homeostasis possibly related to defects in NOD2 surveillance can lead to dysbiosis, which is associated with increased mucosal adherence and consequent bacterial translocation. NOD2 stimulation by MDP also maintains stem cell survival through protection against oxidative stress-mediated cell death, and NOD2 depletion results in reduced stem cell survival and proliferation. Finally, NOD2 variants can cause dysregulation of immune responses in the lamina propria. Defective NOD2 expressed in macrophages and DC are not able to suppress TLR2 signaling leading to overactivation of NF-κB and increased expression of IL-12, IL-1β and IFN-γ, which in turn can lead to damage of the epithelial layer. In addition, DC autophagy-induced T_{reg} cells is reduced favoring a dysregulated inflammation. Abbreviations: ATG16L1: autophagy-related protein 16 like 1; DC: dendritic cell; MDP: muramyl dipeptide; NOD2: nucleotide-binding oligomerization domain 2; NF-κB: nuclear factor kappa B; Th1: T helper 1; TLR: toll-like receptor; Treg: T regulatory cell.

Table 1. Mechanisms of bacterial entry into cells and recognition by NOD1 and NOD2.

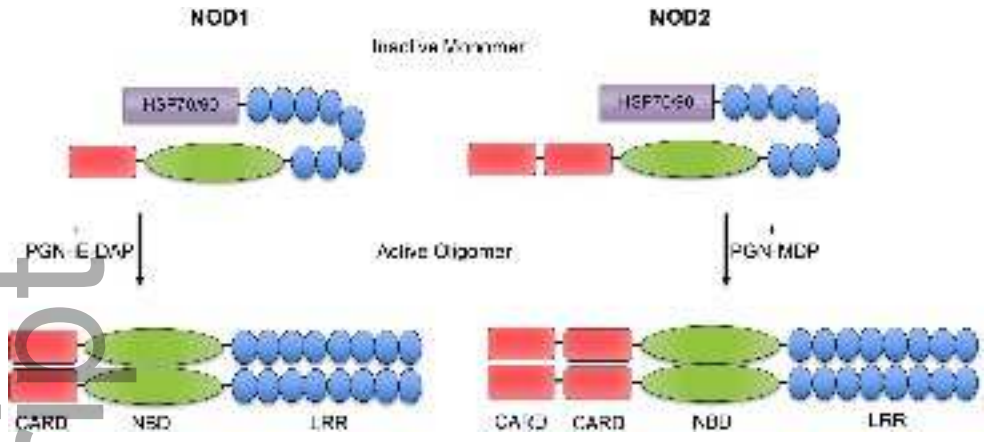
Transport system	Microbe/ligand	Receptor	Outcome(s)	Reference(s)
Uptake of PGN-containing OMVs	*Gram-negative bacteria including H. pylori, P. aeruginosa, V. cholerae, A.actinomycetemcomitans; B. fragilis, N. gonorrhea*	NOD1/2	Autophagy; Migration of NOD1/PGN-OMVs/RIPK2 to early endosome; NF-κB and	(40, 61-63, 66, 181)

			MAPK; IL-8 upregulation; adaptive and immune responses	
SLC15A3/4 peptide transporters	MDP, *Salmonella*	NOD2	MDP transport across endosomal membranes; NF-κB/MAPK activation	(41)
Peptide transporter hPepT1	MDP	NOD2	NF-κB activation in epithelial cell and IL-8 release	(56)
Peptide transporter PepT2	ie-DAP	NOD1	NF-κB/MAPK activation	(57, 58)
Type III secretion system-dependent invasive bacteria	*S. flexneri; S. Typhimurium*	NOD1	NF-κB signaling; RhoA activation; SGT1-dependent IL-8 secretion	(36, 113, 122)
Type IV bacterial secretion system	MDP/*Brucella abortus*	NOD1/2	ER-stress-induced inflammation	(30)
Type VII ESX-1 secretion system	MDP/*M. tuberculosis*	NOD2	Type I IFN expression in Macrophages	(39)

Table 2. Causal genetic variants of NOD1 and NOD2 that are associated with granulomatous autoinflammatory diseases, Crohn's disease, asthma and infectious diseases.

Gene	Blau syndrome/Early Onset Sarcoidosis	Crohn's disease	Asthma	Infectious diseases
NOD1		G796A (255)	ND1+32656 (247) *Rs2975632* *Rs2075822* *Rs2907749* *Rs2907748* (244)	Peptic ulceration in *H. pylori* E266K (256) Cytomegalovirus rs2284358 rs2970500 rs10267377 (91)
NOD2	R334W/Q (223, 227) E383K (226, 257) R587C (227, 228) H480R (258) L469F (223) Y563H (259) E667K (260) T605N (261) G464W (262) E600A (263) D512H (259) E383D (264) C495Y (227) G481D (265) R426H (221, 225) T605P (225, 266) E498G (267) H496L (225, 266) W490L (227, 228) E338D (268) E499_L500delinsV (269) H520Y (268, 270)	R702W (159, 272) G908R (159, 272) Fs1007insC (159, 162, 272) R311W, S431L, R703C, N852S, M863V (272) R138Q, W157R, N289S, D291N, L348V, 558delLG, A612T, A612V, R713C, E843K (231)		Leprosy rs8057431 (273) rs9302752 (274, 275) rs7194886 rs8057341 rs3135499 (275) Tuberculosis Ala725Gly (276) Arg587Arg (277) *H. pylori*-associated lymphoma R702W (278)

N670K (225)
D390V (268)
M513R/T (225, 271)
Q809K (268)



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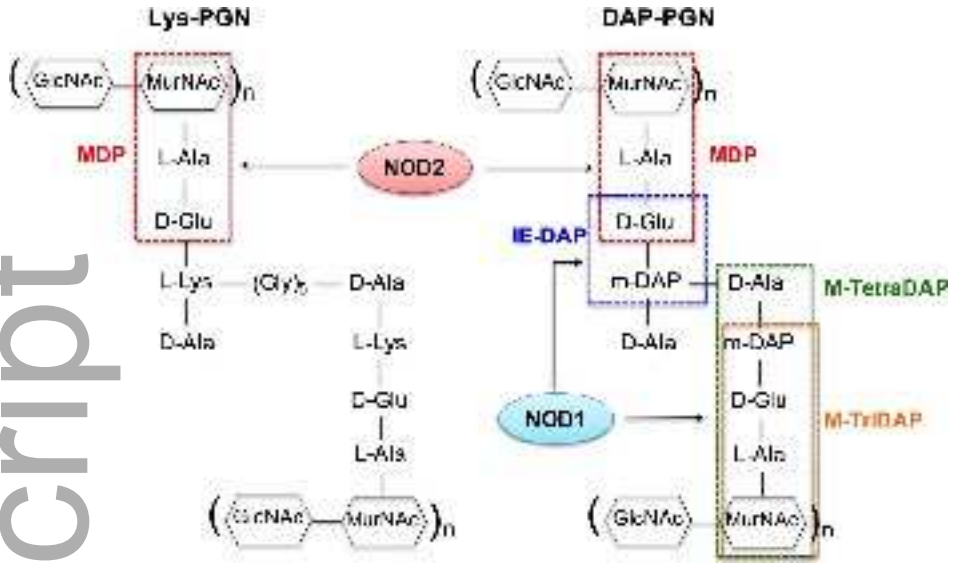

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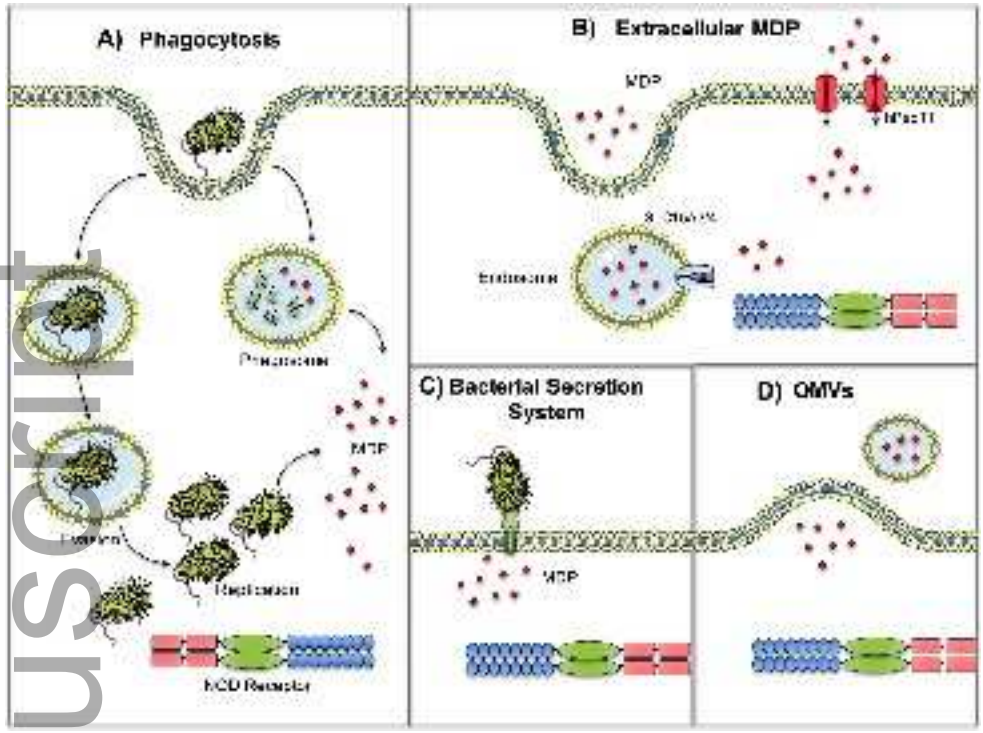

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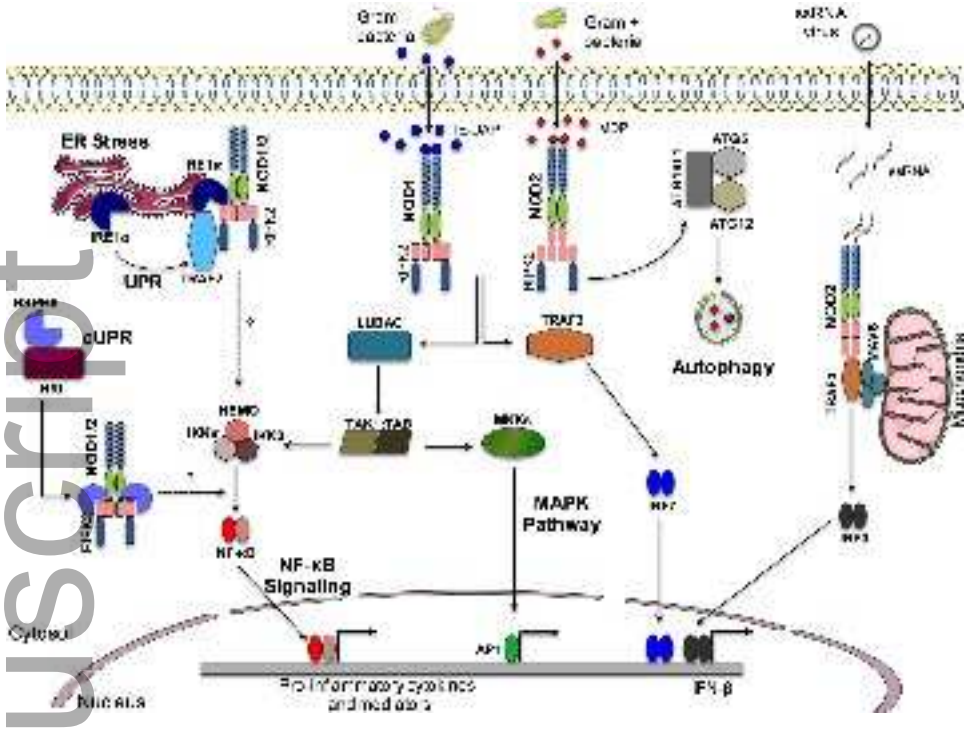

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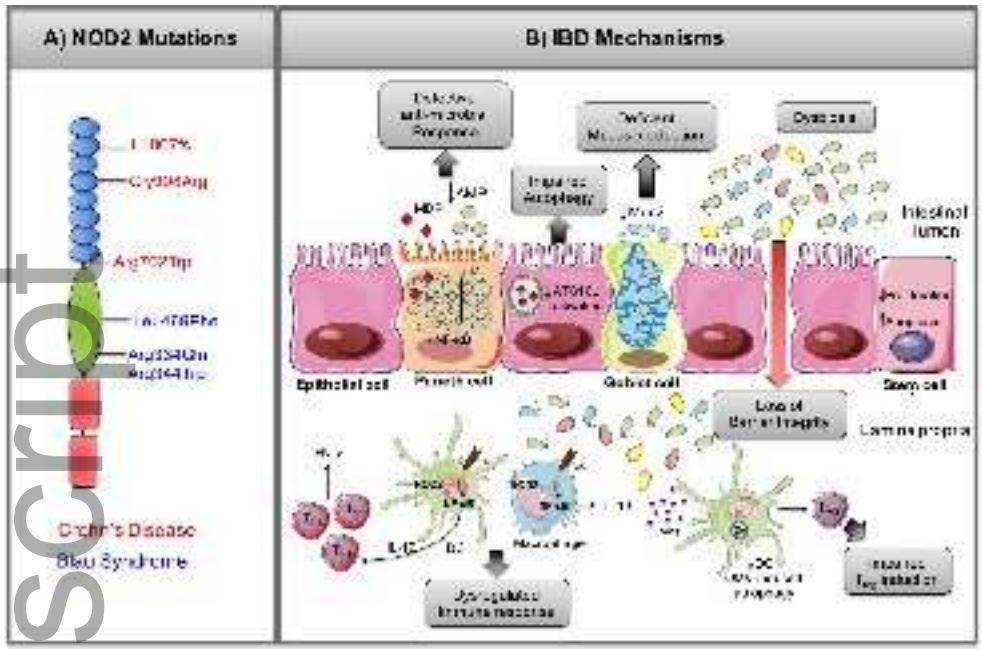

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