Review

NOD2 Signaling and Role in Pathogenic *Mycobacterium* Recognition, Infection and Immunity

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Key Words

Mycobacterium tuberculosis • NOD2 • Sentinel • Innate immune • Tuberculosis

Abstract

The *Mycobacterium* pathogens acquire additional properties to expand their pathogenicity and existence spaces. The interaction between pathogenic *Mycobacterium* components and receptors of host innate immune system is critical for the infection outcome, particularly for the macrophage activation. NOD2 (Nucleotide binding oligomerization domain 2), an intracellular pathogen recognition sensor, attenuates two key putative host bacterial killing mechanisms: interfering the production of TNF- α and inducing resistance to apoptosis. Multiple evidences have shown that NOD2 acts as a non-redundant recognition system of *Mycobacterium*, a successful pathogen with many mechanisms to evade host immunity and leading to insidious disease. Understanding the complex interaction between host and pathogen mediated by NOD2 signaling, might provide novel insight into the pathogenesis of pathogenic *Mycobacterium* and inform the development of more effective vaccines and therapeutics.

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Introduction

The interaction between pathogen and host is crucial for both fates, and is coordinated by T helper1-type pro-inflammatory cytokines, products of phagocytes upon recognition of pathogen-associated molecular patterns of *Mycobacterium* by pattern recognition receptors (PRRs) [1-3]. Both the innate and adaptive immune responses participate in. NLRs are

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intracellular and cytoplasmic sensors that involved in several biological processes, including host defense against pathogen and inflammation [4, 5]. Initial recognition of the bacterium is mediated by pattern recognition receptors (PRR), such as NOD2. NOD2, initially described as a susceptibility gene for Crohn's disease [6, 7] and an intracellular protein containing leucine-rich repeats (LRRs) similar to TLRs, is recognized as an important pattern recognition receptor for *M. tuberculosis* [1, 2]. NOD2 is a cytoplasmatic protein and functions mainly as a NF- κ B pathway activating sensor for bacterial peptidoglycans (muramyl dipeptide, MDP) found in the cell wall of Gram-positive bacteria [8]. Nod2-deficient cells produced less TNF- α after infection with Gram-positive but not Gram-negative organisms, consistent with the greater quantity of PGN in the cell wall of Gram-positive bacteria [9]. In addition, NOD2 seems to be a negative regulator of Toll-like receptor 2-mediated T helper cell type 1 responses and modulates the optimal expression of antimicrobial peptides such as alpha-defensins and pro-inflammatory cytokines and chemokines in the intestinal mucosa [10].

Transinfection studies have shown that expression of NOD2 stimulates NF- κ B activity, a feature that depends on the co-expression and poly-ubiquitination regulation of the adapter protein Rip2 [11]. Both over-expression and siRNA experiments have demonstrated that the production of cytokines (TNF- α and IL-1 β) in response to *M. tuberculosis* and BCG was RIP2- and NOD2- dependent [12]. In addition, macrophages and dendritic cells from NOD2-deficient mice were impaired in the production of pro-inflammatory cytokines and nitric oxide following infection with live, virulent *M. tuberculosis* [13]. Nod2(-/-) mice had a higher bacterial burden in the lungs after infection and succumbed earlier than wild-type controls [14], suggesting that NOD2 is critical to check the growth of *M. tuberculosis* and BCG within mouse macrophages. Furthermore, Recent proof has shown that NOD2 modulate the innate immune response of alveolar macrophages [15], resulting in significant release of TNF- α and IL-6 and the recruitment of autophagosome-associated proteins (IRGM, LC3 and ATG16L1), suggesting that plays a role in the initial control of respiratory *M. tuberculosis* infections. In brief, NOD2 is an important intracellular sensor for the host immune response to various *Mycobacterium* species, including *M. avium* subspecies paratuberculosis(MAP) [16] and *M. leprae* [17], *M. bovis* BCG [14] and *M. tuberculosis*(MTB) [18]. The complex interaction between NOD2 and virulence factor secreted by pathogenic *Mycobacterium* provide novel insight into the pathogenesis of these infectious diseases, inform both the design of improved adjuvant and more effective tuberculosis vaccines.

The Structure and Expression of NOD2

NOD2 is a member of the conserved NLR protein family, previously as a member of CATERPILLER family (also called the NOD-leucine-rich repeat (LRR) protein family) [19]. NLR family proteins share typical tripartite domain structure: a C-terminal ligand recognition domain (LRR), a central NACHT domain (also known as NOD domain) with ATPase activity involved in self-oligomerization and an N-terminus comprised of protein-protein interaction domains, such as caspase recruitment domains (CARDs) or pyrin domains [19, 20]. CARD-containing and pyrin-domain-containing NLRs are functionally associated with both types of molecules activating nuclear factor- κ B (NF- κ B) and/or caspases. Furthermore, mutations in both domain have been linked with inflammatory diseases [21]. NOD2 has a C-terminal LRR, a central NOD and an N-terminal effector domain that contains two CARDs (Fig. 1).

NOD2 expression has been found mainly in antigen-presenting cells (APCs) [20] and intestinal epithelial cells [22, 23], including Paneth cells [24, 25] and monocytes. Serial deletion mutants identified the C-terminal protein domain of NOD2 that is responsible for membrane targeting [26], suggesting that the ligands for these molecules may be derived from microbial components. In fact, multiple evidences have shown that NOD2 recognize peptides that are derived from the degradation of PGN, which is a component of bacterial cell walls [6, 27]. For example, NOD2 interact *M. tuberculosis* PGN through its C-terminal LRR domain to recognize and react to muramyl dipeptide (MDP), a component of bacterial

Fig. 1. The differences of the location and structure between NOD2 and TLRs.

peptidoglycan (PGN)[6, 27], suggesting an intimate role in *Mycobacterium* infection. The expression of NOD2 is regulated by pro-inflammatory cytokines. TNF- α positively regulates NOD2 expression and this is augmented by IFN γ [24]. NF- κ B binding sites in the CARD15 promoter are involved in this response to TNF- α , indicating the ligand-mediated NOD2 activation can trigger NF- κ B and NOD2 is also subjected to self-regulation.

The Recognition of NOD2

Mycobacteria such as *M. tuberculosis* and *M. leprae* are intracellular pathogen residing within specialized compartments and replicating primarily within macrophages. Innate resistance against pathogenic *Mycobacterium* is thought to depend critically on the engagement of pattern recognition receptors on macrophages. After the engagement of pattern recognition receptors (PRRs), microbes harboring pathogen-associated molecular patterns can activate APCs [27-29]. *M. tuberculosis* can deliver virulence factors and immune activators or suppressors through a specialized protein secretion system, namely type VII secretion system [30-32] encoded by the ESX1 locus, to perturb the host membranes [33, 34] and trigger the type I IFN response [35] and activate inflammasome [36]. Type VII system might play a role in the cytosolic recognition of pathogen by NOD proteins due to the bacterial products translocated by it and host membrane damage resulted from these secreted virulence factors. The deletion of Rip2 and the loss of ESX1 can further decrease the IFN β mRNA levels. However, no significant effect can be observed with deleted ESX1 [37], suggesting that the stable ubiquitination of Rip2 is responsible to the recognition of intracellular *M. tuberculosis* and this recognition necessitates the active engagement of *Mycobacterium* secretory apparatus that translocates bacterial products into the cytosol.

The intracellular localization and structure of NOD2 make it highly suitable candidate for the recognition of *Mycobacterium*. Nod2 recognizes muramyl dipeptide (MDP) derived from bacterium peptidoglycan, which consists of a repetitive disaccharide unit of β -1,4-linked

N-acetyl-muramic acid and N-acetyl-glucosamine carbohydrates with cross-linked peptides of varying composition attached to the muramyl moieties [6, 38]. Multiple evidences have shown that NOD2 has been implicated in sensing PGN-derived N-acetyl MDP and to activate the NF- κ B and mitogen-activated protein kinase (MAPK) pathways via polyubiquitination of the RIP2 kinase [18, 27, 39]. The initiation of cytosolic recognition of *M. tuberculosis* might be triggered by the swift TLR2/4-independent polyubiquitination of the Rip2 protein [18]. Nod2-dependent NF- κ B activation pathway required for the stable ubiquitination of Rip2 [18]. In addition, the E2-conjugating enzyme Ubc13, the E3 ubiquitin ligase Traf6, and the ubiquitin-activated kinase Tak1 participate in the Nod2-mediated NF- κ B activation [18]. The proposed scheme for the interplay between *M. tuberculosis* and Nod2 might be like the following: the activation of Nod2 by *M. tuberculosis* infection further triggers the polyubiquitination of Rip2, which in turn proceeds to induce NF- κ B and MAPK activation via the Tbk1 and p38MAP kinase respectively (Fig. 2). Both the enzymes and virulence factors involving in this event might be important novel drug targets for tuberculosis.

However, the recognition of Nod2 was affected by the modifications on these common forms of peptidoglycan in diverse ways [40-42]. In virtually all bacteria, both carbohydrates in the glycan chain are N-acetylated. However, the muramic acid moieties of *Mycobacterium* are N-glycolylated instead [43]. The initial discovery of N-glycolylated muramic acid (Mur-NGlyc)¹ in *Mycobacterium* prompted the hypothesis that it was synthesized from an N-acetylated muramic acid (MurNAc) pre-cursor by the action of a monooxygenase enzyme, generally known as hydroxylase [44]. The rare N-glycolylated modification has been seen only in the peptidoglycan of *Mycobacterium* and five other closely related Actinomycetes (Rhodococcus, Tsukamurella, Gordonia, Nocardia, and Micromonospora) via the action of N-acetyl muramic acid hydroxylase (NamH), which converts their MDP to an N-glycolylated form from N-acetyl muramic acid form [45, 46]. NOD2-dependent immune responses depended on the presence of bacterial namH. The disruption of namH in *M. smegmatis* abrogated NOD2-mediated TNF- α secretion [47], suggesting that N-glycolyl MDP has a greater NOD2-stimulating activity than N-acetyl MDP attributing exceptional immunogenic activity to the *Mycobacterium* cell wall [47]. These may indicate that NOD2 pathway may be exquisitely tuned to detect *Mycobacterium* infections and some components of *Mycobacterium* cell walls may serve as a remarkable adjuvant of anti-tuberculosis drugs.

The NOD2 Signaling Pathway

The NOD2 pathway has been previously implicated in innate immune responses to *Streptococcus pneumoniae*, *Listeria monocytogenes*, and *M. tuberculosis* [3, 48, 49]. Some cross-regulation also exists between TLRs and NOD2 for downstream signaling (Fig. 3). The downstream effector molecule RICK plays a key role in the activation of NF- κ B by NOD2. RICK is a CARD-containing serine/threonine kinase that physically associates with the CARD(s) of NOD2 through CARD-CARD interactions [11]. Transfection of RICK-deficient fibroblasts with NOD2 mutation results in defective NF- κ B activation [50]. However, the RICK-deficient macrophages also have reduced cytokine responses following stimulation with LPS, lipoteichoic acid and PGN [50], indicating that TLR2 and TLR4 might use RICK as a downstream adaptor molecule as well. After the co-stimulation with PGN and MDP, only NOD2-wildtype ACPs were associated with a dose-dependent inhibition of PGN-induced IL-12 production [51], suggesting that MDP-mediated NOD2 negatively regulated TLR2-mediated NF- κ B activation. However, it should be noted that NOD2 can not downregulate TLR4 signaling, another pathway involving RICK [50]. Contamination of NOD2 ligands in the LPS preparations used in these experiments might be the cause of the observed RICK-mediated effects, an artifact of NOD2 signaling instead of TLR4 signaling.

The activation of NF- κ B is the main outcome of NOD2 signaling. The NOD2 mutated epithelial cells will decrease this activation by MDP [6] and abolished NF- κ B subunits translocation to the nucleus in human and mouse APCs [6, 51]. NOD2 activated RICK mediates

Fig. 2. The model of *Mycobacterium* re-cognition via NOD2.

Fig. 3. MDP-mediated NOD2 signaling pathway [78].

K63-linked polyubiquitylation of the inhibitor of NF- κ B, (I κ B)-kinase- γ (IKK γ) at lysine 285 ubiquitylation site [52]. K63-linked poly-ubiquitylation is associated with the activation of the NF- κ B pathway [53]. RICK-IKK γ interaction followed by IKK β phosphorylation and NF- κ B downstream activation, led to the translocation of transcriptional components of NF- κ B into nucleus. No kinase activity of RICK is needed to activate the IKK complex. Either the activation of an E3 ubiquitin ligase that promotes K63-linked polyubiquitylation or the inhibition of an enzyme (such as cylindromatosis protein, CYLD) that de-ubiquitylates proteins modified by K63-linked polyubiquitin by RICK can fulfill above function. Activated NOD2 can also interact with the intracellular molecule GRIM19, a homolog to the NADPH dehydrogenase complex which controls pathogen invasion of intestinal epithelial cells [54]. This interaction might be responsible for the optimal NF- κ B activation [54]. However, the structural basis and the mechanism of this interaction related to NF- κ B activation remain unknown.

The activation of the mitogen-activated protein kinase (MAPK) pathway is another outcome of NOD2 activation. Wild-type instead of NOD2-deficient macrophages stimulated by MDP can activate p38MAPK and extracellular-signal-regulated kinase (ERK) [49, 55]. p38MAP kinase or JNK1/2 activation in Rip2-deficient macrophages were modestly affected by MDP exposure [18], suggesting other CARD-containing adapter proteins might contribute to NOD2-mediated MAP kinase activation. One possibility may be CARD9, as Card9-deficient mice exhibit defects in p38MAP kinase and JNK activation in Nod-stimulated cells. Consistently, MDP-induced MAP kinase responses and cytokine production are impaired in CARD9-deficient cells, whereas MDP-induced NF- κ B activation remains normal [56]. Finally, transfection and immunoprecipitation studies indicate that NOD2 can bind procaspase-1 and induce the secretion of interleukin-1 β (IL-1 β) [57], whose maturation required caspase-1 [58]. These indicate that, NOD2 might bind procaspase-1 through a CARD-CARD interaction, and can bind to RICK and likewise convert the procaspase into a caspase.

NOD2 and Pathogenic *Mycobacterium* Infection

Chronic enteric disease emerges and spreads particularly in individuals with an inherited or acquired susceptibility. Innate immunity recognizes invading microbes and mounts host defensive response aiming to clear the invader. The NOD2 SNP has been implicated in multiple infectious diseases (Table 1), Leprosy, Crohn's disease (CD) and Tuberculosis are the typical representatives of *Mycobacterium* infection.

NOD2 and Leprosy

M. leprae, the causative agent of leprosy, can induce macrophages to release inflammatory cytokines, such as IL-1 β , IL-12, and TNF- α , which are involved in the innate immune response for bacterial elimination and the co-ordination of adaptive immune response [65, 66]. Genome-wide association study (GWAS) on leprosy susceptibility in Chinese population showed that variants of NOD2 gene were significantly associated with the susceptibility to *M. leprae* infection [17]. Recent evidence has shown that an increased NF- κ B activation and expression of TNF- α and IL-1 β in NOD2-transfected cells exposure to *M. leprae* [67]. Interestingly, the activation and expression of these inflammatory cytokine were inhibited by cytochalasin D [67], suggesting that stimulation of NOD2 may be associated with the enhancement of host cytokine production to *M. leprae*.

Table 1. Infectious diseases associated with NOD2 and TLRs

NOD2 Mutations*	Function	Disease	Reference
Arg702Trp, Gly908Arg, Leu1007fsinsCys	Defective NF- κ B activation in response to MDP	Crohn's disease	[6, 27]
Arg334Trp, Arg334Gln, Leu469Phe	Constitutive NF- κ B activation	Blau syndrome	[59-60]
Arg334Trp, His496Leu, Thr605Pro	Constitutive NF- κ B activation	Early-onset sarcoidosis	[61]
Arg702Trp, Gly908Arg, Leu1007fsinsCys	Risk factor for graft-versus-host disease	Graft-versus-host disease	[62-63]
Pro268Ser, Arg702Trp and Ala725Gly	NF- κ B activation and MAPK pathway	Tuberculosis	[64]

NOD2 and Crohn's disease

Crohn's disease (CD) is a systemic disorder with a chronic intestinal inflammation. A susceptibility locus for Crohn's disease has been mapped to chromosome 16 [68] and is tightly linked to markers D16S3396, D16S416 and D16S419, and a site that precisely overlaps with IBD1 [69]. Three additional independent associations for Crohn's disease: a frameshift variant and two missense variants of NOD2, L1007fsinsC, G908R, and R702W, have been genetically associated with susceptibility to Crohn's disease too [7, 69]. In addition, NOD2 variants with altered structure in leucine-rich repeat domain or the adjacent region can force the overexpression of monocyte nuclear factor NF- κ B, suggestive of a novel pathogenesis of Crohn's disease that warrants further investigation.

NOD2 and Tuberculosis

Tuberculosis continues to be one of the most prevalent and deadly infectious diseases [70, 71]. Nearly one-third of the global population is latently infected with *M. tuberculosis* [72]. The epidemic of AIDS has worsened the situation [73, 74]. *M. tuberculosis* can infect and subvert host mononuclear cell (MNCs) responsible for effective host innate and adaptive immune response to sequester, eliminate the pathogen [75]. The increased levels of NOD2 expression in some patients with severe tuberculosis, and the increases in expression levels within peripheral leucocytes following treatment merit further studies in selected patient and control populations [76]. An association between the variant alleles of NOD2 and the subgroup of patients with sputum culture-positive tuberculosis in a Chinese population was established [77], suggestive of the subtle immunity difference might exist between sputum smear-positive cases and sputum smear-negative cases, consisting with previous reported diverse cellular and cytokine responses between patients with different clinical presentations.

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