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Mini-Review

RIP2 activity in inflammatory disease and implications for novel therapeutics

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ABSTRACT

The role of NOD2 and RIP2 in inflammatory disease has been paradoxical. Whereas loss-of-function NOD2 polymorphisms cause CD, a granulomatous disease of the gastrointestinal tract, gain-of-function mutations cause EOS—a granulomatous disease primarily affecting the skin, joints, and eyes. Thus, gain-of-function mutations and loss-of-function polymorphisms cause granulomatous inflammatory disease, only in different anatomic locations. The situation is complicated further by the fact that WT NOD2 and WT RIP2 activity has been implicated in diseases such as asthma, inflammatory arthritis and MS. This article reviews the role that the NOD2:RIP2 complex plays in inflammatory disease, with an emphasis on the inhibition of this signaling pathway as a novel pharmaceutical target in inflammatory disease. *J. Leukoc. Biol.* 94: 927–932; 2013.

NOD2/RIP2 DYSREGULATION IN INFLAMMATORY DISEASE

NOD2 (*CARD15*) is a cytosolic PRR that coordinates innate-immune signaling pathways to help tailor the adaptive immune system to eradicate an offending pathogen. NOD2 acts in tandem with its obligate kinase RIP2 to activate signaling pathways in response to MDP, a component of peptidoglycan from gram-negative and -positive bacteria [1–6]. NOD2 is best known for its association with CD, a chronic, transmural, granulomatous inflammatory disease of the intestinal tract that manifests primarily in the distal ileum, cecum, and colon [7], and one of the largest GWAS of IBD done to date confirmed recently the association of the NOD2 allele with CD [8]. The major CD-associated NOD2 polymorphisms (Leu1007fsinsC, Gly908Arg, and Arg702Trp) occur in the LRR of NOD2 and encode a loss-of-function protein defective in MDP-stimulated NF-κB activation [9–11]. This paradoxically heightened inflammatory state in CD, harboring loss-of-function polymorphisms in NOD2, parallels the hyperinflammatory state seen in primary immunodeficiencies such as chronic granulomatous disease, and it has been hypothesized that CD may, in fact, be a primary immunodeficiency [12]. In support of this, the NOD2:RIP2 complex is known to regulate microbial homeostasis in the intestine, implicating a dysregulated flora and increased mucosal barrier vulnerability that compound a defective innate-immune response [13]. This dysregulated intestinal microbiota has been shown recently to sensitize the colonic mucosa to injury and also to predispose mice to colitis and colorectal cancer [14]. All of these features indicate that loss-of-function NOD2 polymorphisms are consistent with an inadequate defense response upon intestinal breach and that insufficient acute inflammatory processes and heightened mucosal barrier vulnerability exacerbate an inflammatory state that ultimately results in the granulomatous inflammation characteristic of CD.

In contrast to the loss-of-function NOD2 polymorphisms seen in CD, activating mutations of NOD2 within the NACHT domain also cause granulomatous inflammatory disease, albeit in a separate anatomic location. Blau syndrome and EOS are systemic, granulomatous, inflammatory diseases that share a triad of skin, joint, and eye defects [15]. To date, 17 NOD2 variants have been found to be associated with Blau syndrome, of which the majority occur in the NACHT domain of NOD2 [16], and of the subsets of these variants studied in vitro, all show increased basal NF-κB activity [17–19]. Although the

Abbreviations: CARD=caspase activation recruitment domain, CD=Crohn's disease, cIAP=cellular inhibitor of apoptosis, EOS=early-onset sarcoidosis, FDA=U.S. Food and Drug Administration, GWAS=genome-wide association study, IAP=inhibitor of apoptosis, IBD=inflammatory bowel disease, ITCH=homologous to the E6-associated protein carboxyl terminus domain-containing E3 ubiquitin ligase, K63=lysine 63, LRR=leucine-rich region, LUBAC=linear ubiquitin chain assembly complex, MDP=muramyl dipeptide, MS=multiple sclerosis, NACHT=NAIP (neuronal apoptosis inhibitory protein), CIITA (MHC class II transcription activator), HET-E (incompatibility locus protein from Podospora anserina), TP1 (telomerase-associated protein), NEMO=NF-κB essential modulator, NLR=nucleotide oligomerization domain protein 2-like receptor, NOD2=nucleotide oligomerization domain protein 2, RIP2=receptor-interacting protein 2, SMAC=second mitochondrial-derived activator of caspases, Tab=TGF-β-activated kinase 1-binding protein, TAK1=TGF-β-activated kinase 1, XIAP=X-linked inhibitor of apoptosis protein

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mechanism of how exactly hyperfunctional NOD2 leads to these syndromes has not yet been elucidated, Blau syndrome and EOS are, in essence, the genetically converse disorders of CD in that they are caused by hyperfunctioning NOD2 mutations. However, pathophysiologically, they are similar to CD in that they manifest in granulomatous inflammatory disease.

Whereas NOD2 polymorphisms in CD have attracted much attention, polymorphic NOD2 is not sufficient to cause disease. Although 20–25% of CD patients are heterozygous or compound-heterozygous for one of the three mutant NOD2 alleles, CD-associated NOD2 polymorphisms are present in 7–9% of the general population, and most individuals carrying NOD2 polymorphisms never manifest CD; in fact, up to 75% of CD patients and nearly 100% of sporadic sarcoidosis patients have WT NOD2 [9, 20, 21]. Thus, WT NOD2 is far more common in granulomatous inflammatory disease than polymorphic or mutant NOD2. Both WT NOD2 and RIP2 expression are strongly up-regulated by NF-κB [22], and this has led to a recent hypothesis that the feed-forward acceleration of WT NOD2:RIP2 signaling and can contribute to dysregulation in a setting of heightened inflammation [23]. Additionally, in the largest IBD GWAS performed to date, loss-of-function NOD2 polymorphisms were shown to be protective against the development of ulcerative colitis [8], a pathologically and clinically distinct form of IBD.

In vivo disease models also implicate overactive WT NOD2/RIP2 in inflammatory disease. Asthma is a common, chronic lung inflammatory disease, in which airway immune tolerance is essential for preventing allergy-driven asthma. An in vivo mouse study showed that inhalation of the NOD2 agonist MDP inhibits airway tolerance, leading to lung inflammation and susceptibility to allergic asthma [24]. WT NOD1 and NOD2 have been found to be expressed in the synovium of rheumatoid arthritis patients [25], suggesting a potential role for NOD2 in arthritis pathogenesis, and a recent study showed that NOD2 and RIP2-null, but not NOD1-null mice, are protected from antigen-induced arthritis, with a concomitantly lowered, proinflammatory cytokine profile [26]. NOD2/RIP2 is also implicated in a mouse model of MS in that NOD2 and RIP2-null mice are highly resistant to experimental autoimmune encephalomyelitis, the animal model of MS [27].

Whereas there is a noncoding single-nucleotide polymorphism near the RIP2 gene associated with CD, four other genes are in that loci [8], and in physiologic studies, WT RIP2 has also been found to be elevated and essential for NOD2 signaling in a cohort of pediatric CD patients [28]. NOD2/RIP2 also promotes inflammatory dysregulation in infectious disease, such as in the innate-immune response to viral infection. Viral exposure can augment NOD2 activity upon secondary bacterial infection to the point of lethality in mice, and this is attributed, at least in part, to NOD2-induced TNF-α overload [29]. Lastly, human genetics offers in vivo evidence of aberrant WT NOD2/RIP2 in disease pathogenesis. A study of patients deficient in ITCH, a negative regulator of RIP2 [30], showed a striking phenotype, not only of multisystem autoimmune disease but also of organomegaly, failure to thrive, developmental delay, delayed motor development, stunted growth, and dysmorphic features. These 10 patients were the first reported human phenotype of an ITCH deficiency and are homozygous for a premature stop mutation in ITCH (MIM 606409) [31]. As ITCH is a potent negative regulator of NOD2/RIP2 [30], this ITCH defect may implicate hyperfunctional NOD2/RIP2 signaling as a mechanism driving a component of this inflammatory disorder. Thus, in its basal state, NOD2 functions as an intracellular PRR to regulate inflammatory signaling in response to MDP. However, as WT NOD2 expression and activity are hyperactive in a number of inflammatory diseases, there is a strong rationale for pharmacologically inhibiting the NOD2:RIP2 signaling pathway in inflammatory disease (**Fig. 1**).

NOD2/RIP2 SIGNALING

Pharmacologic inhibition of NOD2 or RIP2 requires an in-depth molecular understanding of their functions and their roles in cell signaling. NOD2 is a member of a family of intracellular NLRs that is activated upon exposure to an intracellular breakdown product of peptidoglycan—MDP [1, 2]. NOD2 requires RIP2, a dual-specificity protein kinase, to mediate its downstream signaling, and this active NOD2:RIP2 complex triggers a number of processes, including autophagy, antigen presentation, and MAPK pathway activation. Of these pro-

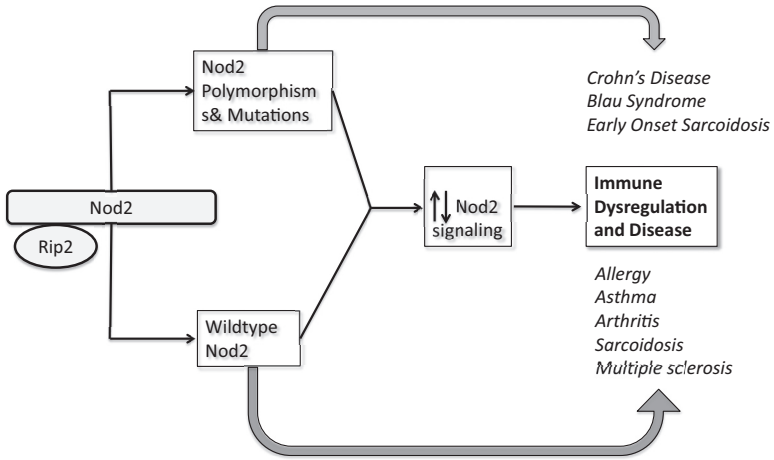

Figure 1. Aberrant NOD2 activity. WT NOD2 and NOD2 polymorphisms/mutations can independently lead to aberrant signaling through RIP2, which can lead to immune dysregulation that is the basis for multiple inflammatory diseases.

cesses, the NF-κB pathway activation has been the most studied and serves as the model for NOD2-influenced signaling [2, 28, 32–36].

NOD2 is comprised of two N-terminal CARD domains: a central NACHT domain and 10 C-terminal LRRs [37]. NOD2 senses MDP through its LRR [38–40], which triggers NOD2 oligomerization and formation of a NOD2:RIP2 complex via homotypic CARD–CARD interactions [37]. This binding then promotes K63-linked ubiquitination of RIP2 by multiple E3 ligases, including cIAP1, cIAP2, XIAP, and TRAF2, -5, and -6 [41–43], recruitment of LUBAC to NOD2 [44], and K63-linked [45, 46] and linear ubiquitination of NEMO [47]. Ubiquitinated RIP2 and NEMO act as a docking site for Tab2 and -3, leading to TAK1 recruitment and activation of the IKK complex, composed of IKKα, IKKβ, and NEMO/IKKγ [48–50]. IKKβ of the activated IKK complex then phosphorylates IκBα, leading to its degradation and the release of NF-κB transcription factors to the nucleus for transcription of NF-κB target genes (**Fig. 2**).

Just as with activation, NOD2/RIP2 deactivation must be tightly regulated. ITCH directly ubiquitinates cIAP1 and RIP2 to inhibit NOD2/RIP2-induced NF-κB activation [30, 51]. ITCH functions in a ubiquitin-editing complex with A20 to down-regulate NF-κB signaling [52]. A20, a dual ubiquitin ligase and deubiquitinase, potently inhibits MDP-activated NF-κB by deubiquitinating the essential positive regulators of NOD2 signaling, such as RIP2, TRAF2, TRAF6, and NEMO [53, 54]. Like ITCH-null mice, A20-null mice show a severe inflammatory phenotype with multiorgan inflammation and hypersensitivity to inflammatory mediators [55, 56]. Additionally, a number of other proteins down-regulate NOD2:RIP2 signaling rapidly. TRAF4 is an E3 ubiquitin ligase that binds NOD2 directly to down-regulate NF-κB signaling [57, 58]. Retinoic acid-induced gene-1 is a RNA helicase viral sensor that also down-regulates NOD2-mediated inflammatory signaling [59]. Additional inhibitors such as Erbin, a leucine-rich repeat and PDZ domain-containing family protein, may regulate cellular localization of NOD2 [60, 61]. Other novel mechanisms of NOD2 down-regulation include tolerance in which NOD2 is ubiquitinated and subsequently proteosomally degraded upon repeated MDP stimulation [62], and competitive ubiquitin binding at the RIP2-binding site of the NOD2 CARD domain [63]. Additionally, a recent genome-wide RNA interference screen in human embryonic kidney 293 cells identified a number of novel, potential negative regulators of the NOD2/RIP2 signaling pathway [64]. Thus, there are multiple potent mechanisms to deactivate the NOD2/RIP2 signaling complex when it is no longer needed, and this down-regulation may be a key mechanism to avoid granulomatous inflammatory disease.

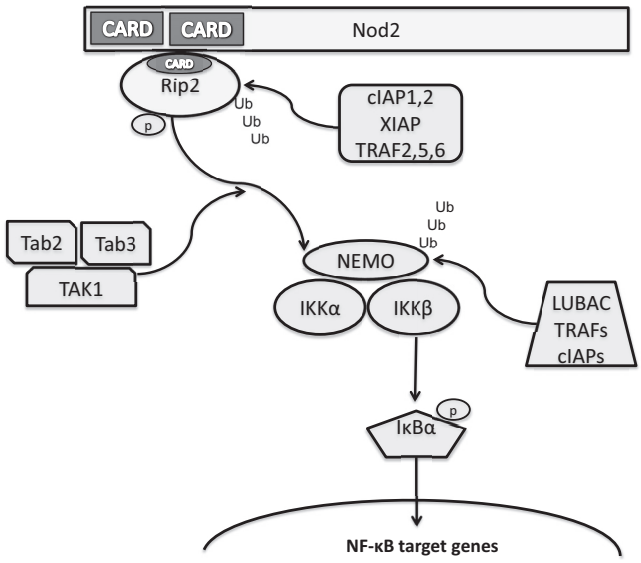

Figure 2. Basic NOD2/RIP2 pathway of NF-κB activation. NOD2 and RIP2 interact via CARD domains upon MDP binding. E3 ligases ubiquitinate RIP2 (Ub), promoting K63-linked and linear ubiquitination of NEMO (IKKγ) of the IKK complex. Ubiquitinated RIP2 and NEMO provide a docking site for TAK1/Tab2/Tab3 complex recruitment, which activates the IKK complex. IKKβ then phosphorylates (P) IκBα, leading to the degradation and release of NF-κB transcription factors to the nucleus.

RIP2 INHIBITION AS A NOVEL, THERAPEUTIC MECHANISM

That RIP2 is indispensable for NOD2 signaling raises the intriguing question of whether its kinase activity can be harnessed to modulate the immune state. The rationale for targeting RIP2 in inflammatory disease is twofold: first, NOD2 can function as a rheostat in modulating the immune state, and secondly, RIP2 kinase activity is necessary for subsets of NOD2 function. The former is suggested in the multiple instances of inflammatory dysregulation and disease stemming from overactive, although WT NOD2/RIP2 activity. The latter is suggested by several studies demonstrating the indispensability of RIP2 kinase activity for subsets of NOD2 function by mediating RIP2 stabilization and the autophosphorylation of tyrosine 474 necessary for NOD2-driven cytokine responses [65–67]. RIP2 antagonists have been used in tissue-culture systems to influence inflammatory output. The p38 inhibitor, SB203580, potently antagonizes RIP2 at nanomolar concentrations, and inhibition of RIP2 kinase activity by SB203580 potently inhibits NOD2-influenced NF-κB activity [68, 74]. The EGFR tyrosine kinase inhibitors gefitinib (Iressa, AstraZeneca, London, UK) and erlotinib (Tarceva, Genentech, South San Francisco, CA, USA) are FDA-approved pharmacologics that potently inhibit RIP2 kinase activity and do so by inhibiting phosphorylation at an essential tyrosine at residue 474 on RIP2 that must be phosphorylated for maximal NOD2 activation [67]. In total, these pharmaceutical studies suggest that RIP2 kinase inhibition can normalize the proinflammatory output of NOD2 and suggest further that RIP2 may be a viable pharmacological target to modulate inflammatory disease.

In addition to FDA-approved therapeutics, such as gefitinib and erlotinib, multiple antagonists against the NOD2/RIP2 pathway are currently under development (**Table 1**). Some are targeted to positive regulators of the NOD2/RIP2 pathway, and some are targeted specifically to NOD2 or RIP2. Others, such as the chromium-containing arene-$Cr(CO)_3$ complexes

TABLE 1. Nod2 Pathway Inhibitors Currently in Development

Kinase inhibitors	SMAC mimetics	XIAP antisense nucleotide	Competitive MDP inhibitor
SB 203580, SB220025, PD169316 (p38 inhibitors; GlaxoSmithKline, Middlesex, UK) [68]	TL-32711 (TetraLogic Pharmaceuticals, Malvern, PA, USA) [69]	AEG35156, AEG40826-2HCl (Aegera Therapeutics, Montreal, Québec, Canada) [70]	Arene-Cr$(CO)_3$ complexes, i.e., AKS-01 (University of Cologne, Germany) [71]
Gefitinib (Iressa; EGFR inhibitor; AstraZeneca) [67]	GDC-0152, GDC-0917 (Genentech, South San Francisco, CA, USA) [69]		
Erlotinib (Tarceva; EGFR inhibitor; Genentech) [67]	LBW242, LCL161 (Novartis, Basel, Switzerland) [72]		
	AT-406 (Ascenta Therapeutics, Malvern, PA, USA) [73]		

[71], are being developed as broad, anti-inflammatory agents and have been found to inhibit MDP/NOD2-mediated NF-κB responses, at least in vitro. Whereas inhibition of RIP2 via SB203580 or Gefitinib is efficacious in in vivo IBD models (refs. [74, 75] and unpublished results), use of these compounds clinically is compromised by their potent, inhibitory effects on p38 and EGFR, respectively. Given this, we have partnered with Oncodesign Biotechnology (Dijon, Cedex, France) to develop and test novel, specific RIP2 inhibitors. Four have been identified with a low-nanomolar RIP2 inhibitory ability in in vitro and cell-culture systems, which are undergoing further developmental activities. Importantly, these compounds show high specificity toward RIP2 when tested against broad kinase panels. In addition to work targeting NOD2 and RIP2 directly, other proteins in the signaling pathway can be targeted. For instance, IAP antagonists [73] and SMAC mimetics [72], which themselves degrade IAPs, are being developed as therapeutics against cancer; however, these cancer drugs may also be efficacious as inflammatory modulators, given that they influence the NOD2/RIP2 pathway [42, 51, 76], underscoring the translational significance of elucidating pathways underlying disease.

RIP2 has a potential, functional reach that extends beyond controlling aberrant NOD2 activity. RIP2 also mediates NOD1 signaling. NOD1, like NOD2, is an intracellular NLR that recognizes specifically γ-D-glutamyl-*meso*-diaminopimelic acid [77], a peptidoglycan moiety of primarily gram-negative bacteria. Like NOD2, NOD1 and RIP2 interact via their respective CARD domains [78], and the association of NOD1 polymorphisms with inflammatory diseases, including asthma [79] and MS [27], suggests an important role of NOD1 in immunomodulation, further reflected in the active development of NOD1 inhibitors [80, 81]. That RIP2 kinase activity is necessary for mediating NOD1 immune functions suggests that RIP2 kinase inhibition may be able to immunomodulate by mechanisms beyond NOD2 signaling. This not only implicates a more expanded, therapeutic reach of RIP2 inhibition but also an increased potential for a combinatorial approach to immunomodulation and heightened specificity of RIP2 therapeutics.

RIP2 clearly plays a critical role in mediating immune signaling, and it is well established that RIP2 plays an indispensable role in transducing NOD2 activation by bacterial components (MDP) into a functional response. RIP2/NOD2 activity in tandem is also implicated in an array of inflammatory dysregulation, from well-circumscribed diseases to exacerbated inflammatory states, highlighting RIP2 modulation as a potentially efficacious mechanism to direct and fine-tune the immune state and ultimately, ameliorate inflammatory disease.

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