

# NOD2 and inflammation: current insights

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**Abstract:** The nucleotide-binding oligomerization domain (NOD) protein, NOD2, belonging to the intracellular NOD-like receptor family, detects conserved motifs in bacterial peptidoglycan and promotes their clearance through activation of a proinflammatory transcriptional program and other innate immune pathways, including autophagy and endoplasmic reticulum stress. An inactive form due to mutations or a constitutive high expression of NOD2 is associated with several inflammatory diseases, suggesting that balanced NOD2 signaling is critical for the maintenance of immune homeostasis. In this review, we discuss recent developments about the pathway and mechanisms of regulation of NOD2 and illustrate the principal functions of the gene, with particular emphasis on its central role in maintaining the equilibrium between intestinal microbiota and host immune responses to control inflammation. Furthermore, we survey recent studies illustrating the role of NOD2 in several inflammatory diseases, in particular, inflammatory bowel disease, of which it is the main susceptibility gene.

**Keywords:** innate immunity, intestinal homeostasis, ER stress, autophagy, inflammatory bowel disease, extraintestinal disease

## Introduction

The human body is constantly in contact with a myriad of microorganisms, either pathogens or commensals. Innate immune system, which provides a first line of defense against many common microbes, is essential for an appropriate tissue homeostasis as well as for common bacterial infections, and its dysfunction leads to infectious, inflammatory and autoimmune diseases.

Innate immune response relies on recognition of evolutionarily conserved structures on the microorganisms, termed pathogen-associated molecular patterns (PAMPs), through a limited number of germ line-encoded pattern recognition receptors (PRRs) present on the host cell surface or in the intracellular compartments.<sup>1</sup> Among the latter, nucleotide-binding and oligomerization domain containing protein 2 (NOD2) is a cytosolic receptor belonging to the nucleotide-binding oligomerization (NOD)-like receptor (NLR) family.<sup>2</sup> NOD2 is able to detect intracellular muramyl dipeptide (MDP), a component of the bacterial wall that is ubiquitously present in bacterial peptidoglycan.<sup>3</sup> Upon activation by ligand, NOD2 mediates innate immune response triggering proinflammatory responses. NOD2 mutation or altered expression has been found in patients with chronic inflammatory disorders such as Crohn's disease (CD), Blau syndrome (BS) and early-onset sarcoidosis (EOS).<sup>4-9</sup> In this review, we summarize the current knowledge about NOD2 functions and regulation, as well as its involvement in chronic inflammatory diseases.

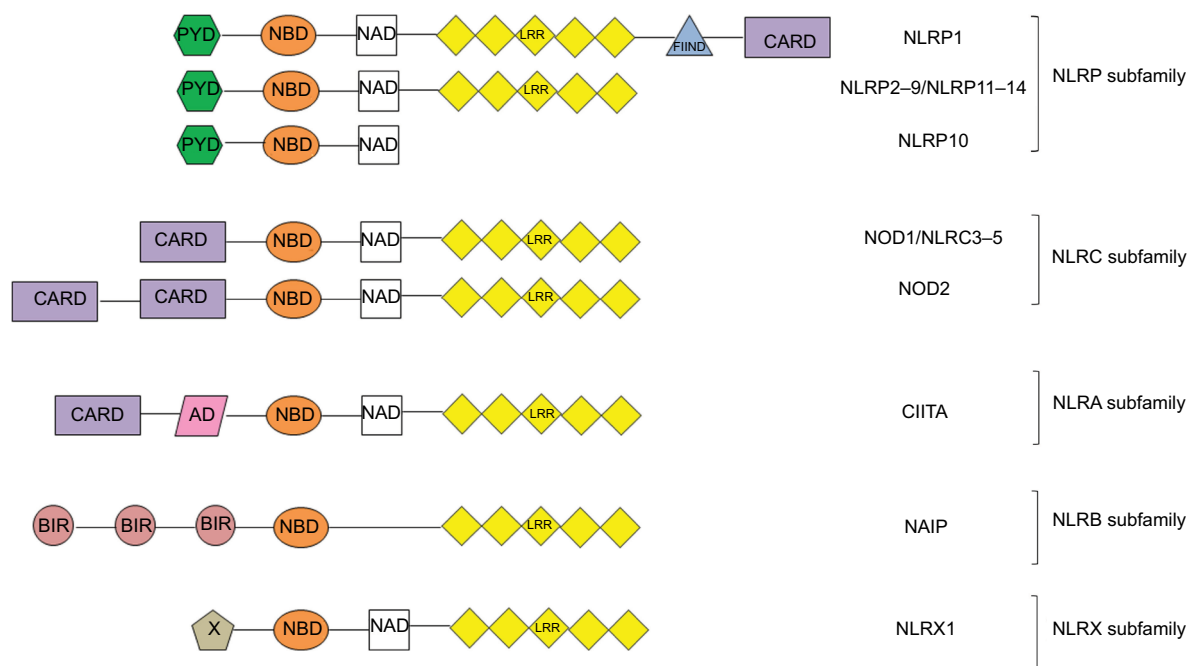
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## The NLR family

The 23 NLR family members, intracellular sensors of PAMPs, share a common organization consisting of a C-terminal leucine-rich repeat (LRR) domain with regulatory and ligand recognition functions, a central nucleotide-binding and oligomerization domain (NBD) and an N-terminal effector-binding domain. The type of effector domain results in the division of NLR proteins into five subfamilies: acidic transactivation domain (NLRA); baculovirus inhibitor repeat, BIR (NLRB); caspase recruitment domain, CARD (NLRC); pyrin domain (NLRP) and NLRX1 that localizes to the mitochondria and has no homology to any known N-terminal domain (Figure 1).<sup>10,11</sup> NOD2 belongs to the NLRC subfamily of NLRs and, after NOD1, has been the second member of the family to be identified.<sup>12</sup> NOD2 receptor, encoded by the *NOD2/CARD15* gene, mapping on chromosome 16q12.1 in humans, consists of 1040 amino acids and has a molecular weight of 110 kDa.<sup>12</sup> It is expressed in monocytes, macrophages, dendritic cells, hepatocytes, preadipocytes, epithelial cells of oral cavity, lung and intestine, with higher expression in ileal Paneth cells and in intestinal stem cells.<sup>13,14</sup> NOD2, like NOD1, is a cytoplasmic protein, although it is recruited in the plasmatic membrane where it detects bacterial invasion at the point of entry.<sup>15</sup>

## NOD2 signaling

The innate immune system is critical for clearing infection and averting excessive tissue damage. NOD2, an intracellular receptor of microbial components derived from bacterial peptidoglycan, contributes to the maintenance of mucosal homeostasis and the induction of mucosal inflammation. Structurally, NOD2 protein is composed of two tandem N-terminal CARDs that function as effector domain and mediate specific homophilic interaction with downstream CARD-containing molecules.<sup>16</sup> On activation by MDP, a cell wall component of both Gram-positive and Gram-negative bacteria, through LRR domain, NOD2 undergoes self-oligomerization and recruitment of the downstream adaptor molecule, the kinase receptor interacting protein 2 (RIP2 also known as RICK, CARDIAK, CCK and Ripk2), via homophilic CARD–CARD interaction.<sup>17–19</sup> Active RIP2 leads to ubiquitination of nuclear factor-kappa B (NF-κB) essential modulator, resulting in activation of IκB kinase (IKK) complex that phosphorylates NF-κB inhibitor-α (IκBα), the inhibitor of transcription factor NF-κB, which translocates to the nucleus and starts transcription of proinflammatory genes, including cytokines, growth factors and factors responsible for stimulation of immune cells.<sup>20,21</sup> RIP2 targets transforming growth factor-β-activated kinase 1, which, through IKK



**Figure 1** Structure of the NLR subfamilies.

**Abbreviations:** AD, atopic dermatitis; BIR, baculovirus inhibitor repeat; CARD, caspase recruitment domain; CIITA, class II major histocompatibility complex transactivator; FIIND, function to find domain; LRR, leucine-rich repeat; NAD, NBD-associated domain; NBD, nucleotide-binding domain; NLR, NOD-like receptor; NLRA, acidic transactivation domain; NLRB, baculovirus inhibitor repeat; NLRC, caspase recruitment domain; NLRP, NLR family pyrin domain; NOD, nucleotide-binding oligomerization domain; PYD, pyrin domain; X, unknown effector domain.

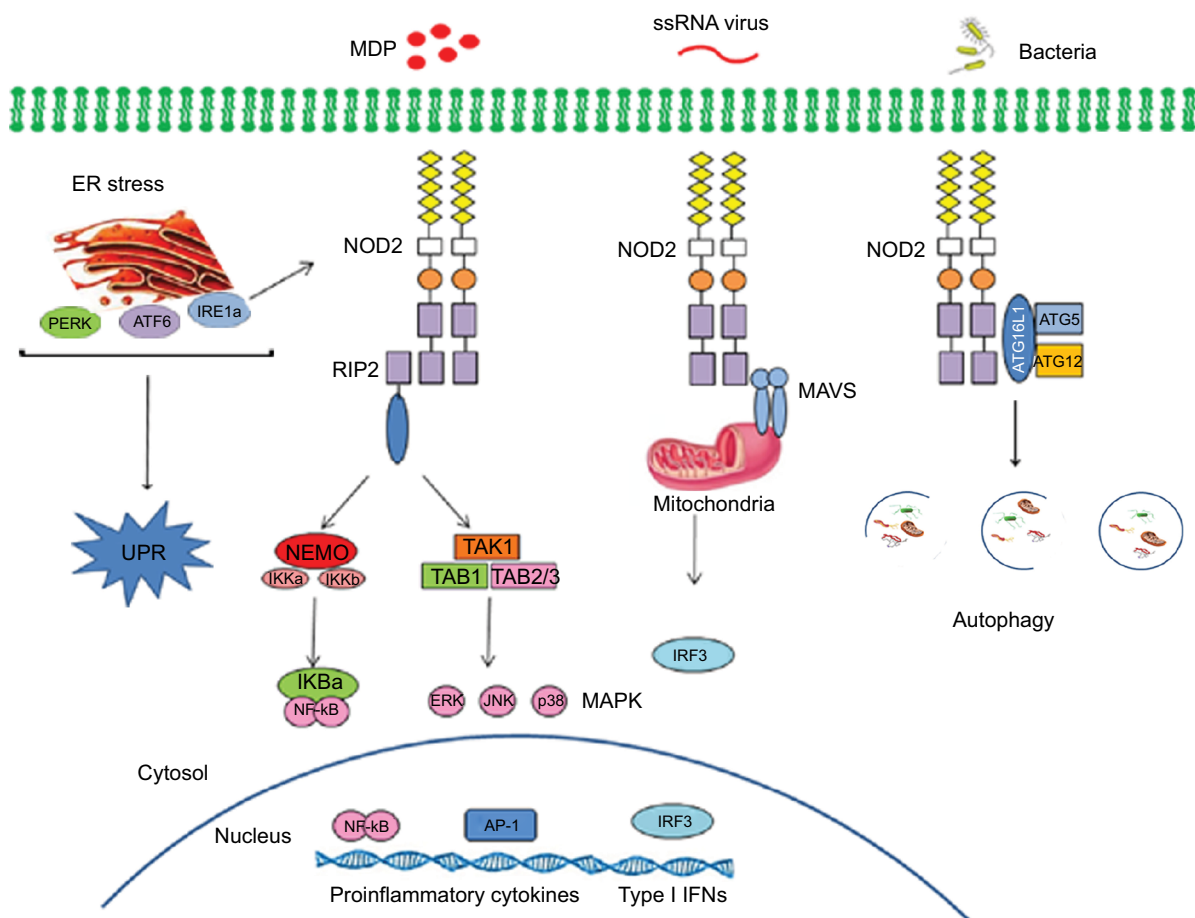
complex, activates MAP kinases and transcription factor Activator Protein 1 involved in cell proliferation, differentiation and apoptosis.<sup>18,22</sup> NOD2 is also known to bind and activate caspase-1, through its CARD domain, and starts interleukin (IL)-1 $\beta$  processing.<sup>23</sup> Moreover, MDP challenge promotes the formation of NOD2–NLR family pyrin domain containing 1 complex that induces caspase-1–dependent IL-1 $\beta$  secretion in response to *Bacillus anthracis* or *Yersinia pseudotuberculosis* infection.<sup>23,24</sup> Recently, NOD2 has been suggested to have a role in the autophagic process due to its interaction with the autophagy protein Autophagy Related 16 Like 1 (ATG16L1), which has been described below in-depth (Figure 2).<sup>25</sup>

## NOD2 regulation

The complexity of NOD2 signaling is underlined by the evidence that many cellular proteins interact with NOD2 directly and regulate positively or negatively its functional activity.<sup>26</sup> Among these, Erbin, Centaurin B1, Angio-Associated

Migratory Cell Protein, Carbamoyl-Phosphate Synthetase 2, Mitogen-Activated Protein Kinase Binding Protein 1 (JNKBP1) and heat shock protein (HSP) 90 have been shown to interact with NOD2 and regulate downstream signaling events.<sup>26–30</sup> Recently, Suppressor of Cytokine Signaling-3 was found to recruit the ubiquitin machinery to NOD2, facilitating its proteasomal degradation.<sup>30</sup> Interferon regulatory factor 4 (IRF4) is another negative regulator of NOD2-dependent NF- $\kappa$ B signaling through inhibition of RIP2 polyubiquitination in human dendritic cells.<sup>31</sup> On the contrary, HSP70 and FERM and PDZ domain-containing protein 2 act as positive regulators of NOD2 signaling: the first one, after binding with NOD2, leads to increase in NF- $\kappa$ B activity and the second one favors NOD2 localization at the plasma membrane.<sup>29,32</sup>

Furthermore, important posttranslational modifications are required to control NOD2 signaling.<sup>33</sup> The E3 ubiquitin ligases Pellino 3 and X-linked inhibitor of apoptosis protein, by ubiquitinating RIP2, are important mediators in the



**Figure 2** Signaling pathways triggered by NOD2.

**Abbreviations:** AP-1, activator protein-1; ATF6, activating transcription factor 6; ATG, autophagy-related genes; ATG16L1, autophagy related 16 like 1; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; IFNs, interferons; IKB, NF- $\kappa$ B inhibitor; IKK, I $\kappa$ B kinase; IRF3, interferon response factor 3; JNK, c-Jun N-terminal kinase; MAVS, mitochondrial antiviral signaling; MDP, muramyl dipeptide; NEMO, NF- $\kappa$ B essential modulator; NOD, nucleotide-binding oligomerization domain; PERK, protein kinase RNA-like endoplasmic reticulum kinase; RIP2, receptor-interacting protein kinase 2; TAB, TGF- $\beta$  activated kinase; TAK1, targets transforming growth factor- $\beta$ -activated kinase 1; UPR, unfolded protein response.

NOD2 pathway and regulators of intestinal inflammation.<sup>34,35</sup> Interestingly, loss of Pellino 3 decreases RIP2 ubiquitination and activation of NF- $\kappa$ B and mitogen-activated protein kinases (MAPKs), while genetic X-linked inhibitor of apoptosis protein loss causes a blunted NOD2 response.<sup>34–36</sup> Tripartite Motif Containing 27 negatively regulates NOD2 by ubiquitination, while the E3-ubiquitin ligase ZNRF4, degrading RIP2, is a negative regulator of NOD2-induced NF- $\kappa$ B activity as well.<sup>37,38</sup> Differently, the ovarian tumor family deubiquitinase OTULIN was shown to dampen NOD2 signaling by increasing NF- $\kappa$ B transcription.<sup>39</sup>

A recent study shows that leucine-rich repeat kinase 2, whose polymorphisms have been associated with CD, leprosy and familiar Parkinson's disease, is a new RIP2-positive regulator by enhancing RIP2 phosphorylation upon NOD2 activation.<sup>40</sup>

NOD2 signaling is known to be regulated by cytoskeleton elements also: the intermediate filament protein vimentin has been recently shown to interact with NOD2, in response to MDP, and affect NF- $\kappa$ B induction.<sup>41</sup>

Large evidence supports the role of several microRNAs, including mir-320, miR-192, miR-122, miR-512, miR-671 and miR-495, as new important NOD2 regulators.<sup>42,43</sup>

## NOD2 genetics

Hereditary polymorphisms in the gene encoding NOD2 have been associated with an increasing number of chronic inflammatory diseases, such as CD, BS and EOS.<sup>6–8</sup>

In particular, the three main NOD2 polymorphisms, R702W (Arg702Trp), G908R (Gly908Arg) and L1007fsinsC, are highly associated with susceptibility to CD.<sup>6,7,44</sup> R702W and G908R mutations are single amino acid changes within the LRR domain, whereas the L1007fsinsC mutation is caused by a deletion producing a reading frameshift that leads to the loss of 33 amino acids.<sup>6,7</sup> CD-associated mutations result in NOD2 loss of functions, with a reduced responsiveness to MDP, enabling invasion of bacteria and abnormal mucosal immune response, which culminates in chronic intestinal inflammation.<sup>5–7</sup>

The variants in the NBD and in between the NBD and LRR are associated to BS, EOS and NOD2-associated auto-inflammatory disease, respectively.<sup>8,9,45,46</sup>

At least 17 different mutations have been identified in the NBD domain of NOD2,<sup>45</sup> of which the following four missense mutations are the most abundant: Arg334Glu (R334Q), Arg334Trp (R334W) and Leu469Phe (L469F), which together account for 80% of the cases, and Glu383Lys (E383K) (5% of cases).<sup>47</sup> Other NOD2 mutations, like the

Arg314Glu (R314Q) polymorphism that codes for a truncated form of the protein, have been described most rarely.<sup>8</sup> These mutations are supposed to be gain of function, causing excessive NF- $\kappa$ B and MAPK activation.<sup>9</sup>

## NOD2 functions

### NOD2 and intestinal microbiota

Humans are colonized by a collection of microbes, the largest numbers of which reside in the distal gut. The constant exposure of the intestinal tissue to gut microorganisms maintains the mucosa in a state of physiological inflammation, which balances tolerogenic and proinflammatory type responses to maintain homeostasis.<sup>48</sup> Several studies highlight the essential role that NOD2 plays in maintaining the equilibrium between microbiome and host immune responses.<sup>49–51</sup> An imbalance in this relationship results in dysbiosis, whereby pathogenic bacteria prevail on commensals, causing damage in the intestinal epithelial barrier as well as allowing bacterial invasion and inflammation.<sup>52,53</sup> A negative feedback loop between NOD2 and commensal bacteria function has been described, whereby commensal bacteria promote NOD2 expression, which in turn prevents their over-expansion.<sup>52</sup> Since NOD2 is an intracellular microbial sensor for gram-positive and gram-negative bacteria, it has been proposed that its deficiency or mutations can contribute to the modification of microbial composition and disease development in animal models.<sup>18,51,52</sup>

*NOD2*-deficient mice show a reduced number of intestinal intraepithelial lymphocytes impairing the epithelium integrity and leading to altered immune response to the resident microbiota.<sup>54</sup> Moreover, *NOD2*- and *RIP2*-deficient mice show increased sensitivity to dextran sulfate sodium-induced colitis and colonic adenocarcinoma due to dysbiosis, which is transmitted to wild-type (WT) mice through the microbiota.<sup>55</sup> *NOD2* is also a critical regulator of microbiota in the small intestine.<sup>13</sup> Indeed, *NOD2* is highly expressed in Paneth cells, specialized cells located at the base of the intestinal crypts of Lieberkuhn, which are responsible for the ileal microbiota by secreting antimicrobial compounds, in response to bacterial products, including MDP, the agonist of *NOD2*.<sup>13,18,56</sup>

There is evidence that *NOD2* mutations increase the susceptibility to abnormal ileal inflammation. *NOD2*-deficient mice display Paneth and goblet cell dysfunction that promotes larger loads of bacteria in the ileum, including *Bacteroides vulgatus*, and excessive interferon- $\gamma$  (IFN $\gamma$ ) production.<sup>53,57</sup>

However, several studies do not support the evidence of a relationship between *NOD2* mutations and altered microbiota composition, suggesting the contribution of other factors independent of the genotype, such as maternal microbiota

transmission or environmental causes, but results are still controversial.<sup>58–60</sup> NOD2 participates in the recognition of a subset of pathogenic microorganisms that are able to invade and multiply intracellularly, such as *Campylobacter*, *Citrobacter*, *Escherichia*, *Helicobacter*, *Listeria*, *Mycobacteria*, *Pseudomonas*, *Staphylococcus*, *Yersinia* and other species.<sup>51,61,62</sup> In a murine skin *Staphylococcus aureus* infection model, *Nod2*<sup>-/-</sup> mice show skin lesions and increased bacterial numbers in the skin, compared to WT mice.<sup>63</sup> Moreover, NOD2-deficient mice show a higher fecal bacterial load after *Citrobacter rodentium* infection, besides increased susceptibility to enteric *Salmonella* spp. and an impaired activation of Th17 cells after *C. rodentium* or *Salmonella* spp. exposure.<sup>64–66</sup> Interestingly, NOD2L1007fsinsC mutants show a lower reactive oxygen species production and a reduced protection against bacterial invasion.<sup>67</sup>

NOD2 upregulation following vitamin D treatment results in increased killing of pneumococci in patients with frequent respiratory tract infections.<sup>68</sup> Bacterial lipoprotein-tolerogenic macrophages show improved NOD1/NOD2-dependent bactericidal activity to *St. aureus* and *Salmonella typhimurium*.<sup>69</sup>

NOD2 cooperates with other proteins for defense against pathogens, such as the autophagic protein ATG16L1 and the oxidase dual oxidase 2 that represents a major source of NOD2-dependent bactericidal reactive oxygen species generation.<sup>70</sup>

### NOD2 in innate and adaptive immunity

NOD2 drives the innate inflammatory response to bacteria and viruses through the activation of NF- $\kappa$ B, MAPK and caspase-1 pathways, which results in increased expression of proinflammatory factors, including IL-1 $\beta$ , tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), IL-6, IL-12p40, CC-chemokine ligand 2, the neutrophil chemoattractants CXC-chemokine ligand 8 (also known as IL-8), CXC-chemokine ligand 2 as well as various antimicrobial agents, as defensins, and in recruitment and priming of neutrophils, inflammatory monocytes and dendritic cells.<sup>64,71–73</sup>

NOD2 also activates other signaling pathways. Upon binding the bacterial ligand, NOD2 induces recruitment of RIP2, which binds TNF receptor-associated factor (TRAF) 3 that activates the TANK binding kinase 1 and IKK $\epsilon$ , which phosphorylates and activates regulatory interferon response factor 3 (IRF3). Activated IRF3 passes into the nucleus, where it binds with the molecules of IFN-stimulated response elements inducing the IFN gene expression of type I.<sup>74,75</sup>

Moreover, NOD2 triggers Notch1 signaling response. Crosstalk between NOD2 and its novel downstream effector Notch1-PI3K in the macrophages contributes to macrophage survival, reducing TNF $\alpha$ /IFN $\gamma$ -induced apoptosis, and modulates the expression of IL-10 and a battery of genes associated with anti-inflammatory functions.<sup>76</sup>

In addition to its main role in innate immunity, NOD2 is capable of activating the adaptive immune system; indeed, it is a key driver of T helper (Th) 2-type immunity resulting in IL-4 and IL-5 production.<sup>77</sup> Full Th2 induction upon *Nod2* activation is dependent on both thymic stromal lymphopoietin production by the stromal cells and the upregulation of the co-stimulatory molecule, OX40 ligand, on the dendritic cells.<sup>77</sup> Several studies have shown that co-stimulation with NOD2 and toll-like receptor (TLR) agonists induces a synergistic production of Th1-associated cytokines in different types of cells, although the mechanism is still unclear.<sup>78,79</sup>

Finally, MDP-induced NOD2 activation has been shown to promote the development of Th17 cells and the consequent production of IL-17A, IL-17F, IL-21 and IL-22.<sup>80,81</sup>

### NOD2 and antiviral response

The innate immune system detects viral infections through the recognition of virus-associated PAMPs, such as genomic DNA, RNA or dsRNA produced in infected cells, which are recognized by the PRRs expressed in innate immune cells.<sup>82</sup> After recognition of the viral components, PRRs activate an antiviral response that results in the production of type I IFNs, proinflammatory cytokines, eicosanoids and chemokines and subsequent induction of adaptive immune response.<sup>82</sup> Current literature evidences that NOD2 is also able to control virus infections.<sup>83,84</sup> Indeed, the antiviral SB 9200, a novel first-in-class oral modulator of innate immunity with a broad-spectrum activity in vitro and in vivo against RNA viruses including hepatitis C virus, norovirus, respiratory syncytial virus and hepatitis B virus, is also believed to act via NOD2 pathway.<sup>85</sup> Furthermore, infection with human cytomegalovirus results in significant induction of NOD2 expression, activating downstream NF- $\kappa$ B and IFN pathways.<sup>84</sup> Besides, following recognition of a viral ssRNA genome, NOD2 uses the adaptor mitochondrial antiviral signaling protein to activate IRF3; interestingly, NOD2-deficient mice fail to produce IFN efficiently. Similar roles for NOD2 are observed in response to influenza A and parainfluenza viruses.<sup>75</sup>

NOD2-induced triggering of NF- $\kappa$ B and IRF3 in response to viral infection is also interesting since it has been shown that a coordinated activation of NF- $\kappa$ B and IRF3 pathways synergistically promotes optimal IFN expression, including



IFN- $\beta$ , and hence the antiviral resistance.<sup>86</sup> Moreover, a regulatory role of NOD2 in enhancing the function of RNase-L through the binding with 2'-5'-oligoadenylate synthetase type 2, a dsRNA binding protein, has been described as an additional strategy to induce an innate immune response to control bacteria and viruses.<sup>87</sup>

## NOD2 and autophagy

Autophagy represents a cellular stress response that plays key roles in physiological processes, such as innate and adaptive immunity, adaptation to starvation, degradation of misfolded or aggregated proteins or damaged organelles and elimination of intracellular pathogens, in order to promote cellular survival. The role of NOD1 and NOD2 in autophagy is a recent discovery and still under debate.

Travassos et al suggested that, by a mechanism independent of the adaptor RIP2 and transcription factor NF- $\kappa$ B, NOD1 and NOD2 recruited the autophagy protein ATG16L1, an essential component of the autophagic machinery, to the plasma membrane at the bacterial entry site.<sup>25</sup> Moreover, in cells homozygous for the CD-associated NOD2 frameshift mutation, mutant NOD2 failed to recruit ATG16L1 to the membrane and wrapping of invading bacteria by autophagosomes was impaired.<sup>25</sup> Furthermore, knockdown of ATG16L1 resulted in specific upregulation of NOD2 response, establishing a role of ATG16L1 as a negative regulator of the NOD2/RIP2 pathway.<sup>88</sup> NOD2-mediated autophagy is crucial to hold intramucosal bacterial burden and limit intestinal inflammation.<sup>89-91</sup>

Interestingly, ATG16L1 and NOD2 single nucleotide polymorphisms are both implicated in increased susceptibility to CD.<sup>91-94</sup>

More recently, other authors have provided evidence of an alternative mechanism of NOD2-dependent autophagy activation which requires RIP2. Cooney et al have shown that NOD2 triggering by MDP induces autophagy in dendritic cells and this effect requires RIP2.<sup>95</sup> Anand et al demonstrated that macrophages deficient in the TLR2 and NOD/RIP2 pathway display defective autophagy induction in response to *Listeria monocytogenes*.<sup>96</sup> Homer et al have shown a dual role for RIP2 tyrosine kinase activity in NOD2-dependent autophagy: RIP2 both sends a positive autophagy signal through activation of p38 MAPK and relieves repression of autophagy mediated by the phosphatase PP2A.<sup>97</sup>

## NOD2 and endoplasmic reticulum (ER) stress

The ER stress, activated by accumulation of unfolded or misfolded proteins and microbial infections, triggers a host

response known as the unfolded protein response (UPR), which involves the activation of three transmembrane receptors: activating transcription factor 6, protein kinase RNA-like ER kinase and inositol-requiring enzyme 1 $\alpha$  (IRE1 $\alpha$ ). Once activated, IRE1 $\alpha$  recruits TRAF2 to the ER membrane to initiate inflammatory responses via c-Jun N-terminal kinase (JNK) pathway and NF- $\kappa$ B.<sup>98</sup> NOD2 was found to transduce ER stress signals to elicit inflammation.<sup>99,100</sup> Indeed, TRAF2 has been shown to interact with NOD1 and NOD2 to orchestrate this inflammatory branch of UPR, which also requires the adaptor protein RIP2.<sup>98</sup> However, the mechanism by which ER stress, and more specifically IRE1 $\alpha$ , activates NOD1/NOD2 signaling is still unclear. Recent work provides evidence that the ER stress inducers thapsigargin and dithiothreitol trigger production of the proinflammatory cytokine IL-6 in a NOD1/2-dependent fashion.<sup>100,101</sup> In vivo experiments confirmed the in vitro observations by showing that systemic proinflammatory responses induced by thapsigargin administration were blunted in *NOD1<sup>-/-</sup>NOD2<sup>-/-</sup>* mice.<sup>100</sup>

Several UPR-related genes have been identified as inflammatory bowel disease (IBD) risk loci.<sup>102,103</sup> In particular, new evidence has linked the UPR and autophagy in Paneth cells to the development of CD-like transmural ileitis.<sup>104</sup> The genetic convergence of genetic polymorphisms on innate immune pathways, such as NOD2, autophagy and ER stress, may open novel therapeutic options for the treatment of intestinal inflammation.<sup>105,106</sup> The involvement of ER stress and NOD2 in chronic inflammatory diseases, including IBD and type 2 diabetes, has important implications for understanding the pathogenesis and for the management of these diseases.<sup>107,108</sup>

## NOD2 and IBD

IBD is a group of chronic multifactorial disorders that includes CD, characterized by transmural inflammation that can affect any region of the gastrointestinal tract and ulcerative colitis that results in inflammation and ulcers of the colon and rectum. The etiology of IBD has yet to be fully elucidated; however, it is postulated that it is the result of an unbalanced crosstalk between gut luminal content and the mucosal immune system in genetically susceptible hosts.<sup>109</sup>

Recent genome-wide association studies have revealed 163 susceptibility loci for IBD.<sup>110</sup> *NOD2* was the first gene identified as a risk factor for ileal CD.<sup>6,7</sup>

Three *NOD2* polymorphisms in the LRR region are directly associated with CD, of which the most known is the frameshift mutation (L1007fs), whereas the other two are missense mutations (R702W and G908R).<sup>6,7</sup> It is postulated that the LRR domain of CD-associated variants is likely

to be impaired, possibly to various degrees, in recognizing microbial components and/or in physiologically inhibiting NOD2 dimerization, thus resulting in the inappropriate activation of NF- $\kappa$ B in monocytes, bacterial clearance and impairment of intestinal permeability.<sup>6,111,112</sup> Thus, individuals who are heterozygous for NOD2 variants have a 2–4-fold increased risk of developing CD, whereas homozygous variants have an additional risk of 20–40-fold.<sup>6,7,113</sup> However, many NOD2 variant carriers do not develop the disease, suggesting that other factors are involved in disease onset.<sup>111,112</sup> Similarly, *NOD2*-knockout mice lack symptoms associated with spontaneous intestinal inflammation.<sup>114</sup> Nevertheless, *NOD2*-deficient mice demonstrate defects in mediating antibacterial responses, such as increased systemic *Listeria* colonization, suggesting decreased antimicrobial peptides in their intestine.<sup>18</sup> Moreover, *NOD2*-deficient mice show altered gut permeability.<sup>49</sup> This evidence is also supported by Amendola et al who attributed the absence of spontaneous colonic inflammation in *NOD2*-deficient mice to permeability changes: the latter may increase the exposure of dendritic cells to factors, such as TLR ligands, that influence T-reg cell development and subsequent changes in the microbiota.<sup>115</sup>

Homozygous/compound heterozygous carriage of the CD-associated NOD2 variants has been reported to be significantly associated with ileal disease involvement, stricturing/stenosing disease behavior and 2–3 years earlier age of disease onset compared to NOD2 WT CD patients.<sup>116,117</sup> Ileal CD patients with NOD2 L1007fs mutation showed a reduced release of  $\alpha$ -defensin from Paneth cells.<sup>118,119</sup>

Interestingly, the reduction of  $\alpha$ -defensin release seems to be a secondary effect of the NOD2 knockdown, causing excessive inflammation and loss of Paneth cell. However, the question remains controversial.<sup>120</sup>

Notably, CD-associated NOD2 variants are defective in ATG16L1 recruitment and exhibit an altered autophagy in a cell type-specific manner.<sup>91</sup> Furthermore, in patients with ileal CD, mutations in autophagy genes other than in NOD2 lead to an impaired secretion of Paneth cell-derived  $\alpha$ -defensins with a deficiency in clearance of internalized bacteria.<sup>119–122</sup> Therefore, the combination of defective innate immune responses by NOD2 and ineffective bacterial clearance by autophagy could together be responsible for CD development and progression.

## NOD2 and extraintestinal diseases

A consistent issue in human genetics is that genes implicated in one disorder could potentially increase susceptibility in other related disorders. It has been shown that three major

missense mutations in the NBD domain of NOD2, R334Q, R334W and L469F are involved in an extremely rare, monogenic dominant disorder characterized by granulomatous inflammatory arthritis, uveitis and dermatitis, known as BS.<sup>45,123,124</sup> The latter is related to gain-of-function mutations as opposed to CD mutations which appear to be recessive and are characterized with respect to NF- $\kappa$ B activation by a loss of function.<sup>44</sup> More rarely, the NOD2 R314Q polymorphism, coding for a truncated form of the protein, has also been found to be associated with BS.<sup>8</sup>

NOD2 mutations, leading to increased activity of NF- $\kappa$ B, have been associated to EOS, a multisystemic granulomatous disease characterized by arthritis, uveitis and cutaneous involvement.<sup>125</sup> NOD2 mutations and similar clinical and histologic characteristics suggest a link between EOS and BS.<sup>9,47</sup>

Increased NOD2 expression has been found in patients with rheumatoid arthritis, a chronic inflammatory disorder that affects the joints, causing structural deformities.<sup>126</sup> Upon stimulation with MDP, peripheral blood mononuclear cells isolated from rheumatoid arthritis patients produced high amounts of TNF $\alpha$ , IL-8 and IL-1 $\beta$ , while NOD2 downregulation significantly decreased proinflammatory cytokines, NF- $\kappa$ B, TRAF6 and IKK levels.<sup>127</sup>

Clinical and experimental studies have recently shown a role of NOD2 in cardiovascular diseases inducing vascular inflammation and severity of atherosclerosis, the most common pathologic process of coronary artery and cerebrovascular disease.<sup>128</sup> NOD2 has been localized in inflamed areas of atherosclerotic lesions and is overexpressed in endothelial cells delimiting the lumen of diseased vessels.<sup>129</sup> Moreover, NOD2-mediated IL-6, IL-8 and IL-1 $\beta$  production induces vascular inflammation and promotes expansion of the lipid-rich necrotic areas.<sup>130</sup>

NOD2 mRNA has also been found to be highly expressed in bronchoalveolar lavage and in peripheral blood mononuclear cells from patients with Behcet's disease, a rare disorder with unknown etiology, characterized by a systemic vasculitis and pulmonary manifestations.<sup>131,132</sup> A recent study has shown that NOD2 aggravates myocardial ischemia/reperfusion injury by inducing cardiomyocyte apoptosis and inflammation through JNK, p38MAPK and NF- $\kappa$ B signaling in mice and has suggested NOD2 as a potential target for the treatment of the disease.<sup>133</sup> Furthermore, NOD2 has been reported to be involved in IL-6, IL-8 and monocyte chemoattractant protein-1 production induced by the invasion of human aortic endothelial cells by *Streptococcus mutans*, the primary etiologic agent of dental caries, associated with the development of cardiovascular disease.<sup>134</sup>

NOD2 variants have been associated with the systemic autoinflammatory disease (NOD2-associated autoinflammatory disease), a genetically complex multisystem disorder characterized by periodic fever, dermatitis, arthritis and gastrointestinal and sicca-like symptoms, but with a phenotypic and genotypic profile distinct from CD.<sup>135</sup>

NOD2 polymorphisms have been also related to atopic dermatitis, a chronically relapsing inflammatory skin disease associated with basophil infiltration and an exacerbation of inflammation by *St. aureus*.<sup>136–138</sup>

Recent literature describes a central role of NOD2 in susceptibility to obesity and metabolic dysfunction.<sup>139</sup> *NOD2*-deficient mice show increased bacterial adherence to the intestinal mucosa and bacterial infiltration in metabolic tissues, such as hepatic and adipose tissue, exacerbating inflammation and insulin resistance.<sup>140</sup> Moreover, *NOD2*<sup>−/−</sup> BALB/c mice have shown susceptibility to obesity, hyperlipidemia, hyperglycemia, glucose intolerance, increased adiposity and hepatic steatosis, as compared to WT mice.<sup>141</sup>

Finally, *NOD2*-increased expression has been observed in patients with active Vogt–Koyanagi–Harada disease, a rare granulomatous inflammatory disease that affects pigmented structures, promoting proinflammatory cytokine production and Th1 and Th17 cells stimulation.<sup>142</sup>

## NOD2 and cancer

*NOD2* has been associated to cancer development. Data from animal models indicate that *NOD2* deficiency leads to dysbiosis resulting in increased risk of colitis and colitis associated colorectal cancer.<sup>143</sup> A recent study in *NOD2*- or *RIP2*-deficient mice shows an increased epithelial dysplasia following dextran sulfate sodium-induced colitis, suggesting a *NOD2*-protective role in cancer development via regulation of gut bacterial equilibrium.<sup>55</sup>

Emerging evidence in human studies implies that several *NOD2* polymorphisms may influence individual susceptibility to cancer.<sup>143,144</sup> However, the results are still inconclusive due to confounding genetic, bacterial and environmental factors that may alter variant allele penetrance or the differences in sample size, geographic variation and genotyping methods. In general, *NOD2* gene polymorphisms are associated with altered risk of gastric, colorectal, breast, ovarian, prostate, testicular, lung, laryngeal, liver, gallbladder, biliary tract, pancreatic, small bowel, kidney, urinary bladder cancer, skin cancer, non-thyroid endocrine tumors, lymphoma and leukemia.<sup>145</sup> In particular, the three most common *NOD2* polymorphisms, rs2066844 C/T (R702W), rs2066845 C/G (G908R) and rs2066847 (3020insC), have been associated

with increased risk of gastrointestinal cancer.<sup>146</sup> Furthermore, *NOD2* has also been involved in the development of gastric cancer induced by *Helicobacter pylori*.<sup>144</sup>

## Conclusion

The involvement of *NOD2* in the pathogenesis of several genetic diseases indicates that this protein is a key regulator of immune and inflammatory responses. Extensive studies have evidenced a fundamental role of *NOD2* in maintaining the equilibrium between bacteria, epithelia and innate immune response of the host. This protective function is lost in case of *NOD2* mutations, resulting in exacerbated inflammation and the onset of various diseases. On the other hand, inappropriate activation of WT *NOD2* is reported in many chronic inflammatory diseases, resulting in continuous production of proinflammatory mediators, but the exact mechanism underlying this *NOD2* dysregulation is not yet well established. Recent literature has shifted the interest from genetic to epigenetic control and to interactions with other innate immune pathways such as autophagy and ER stress. Many questions remain unanswered, including the relation between *NOD2* mutations and microbiota and the understanding of the processes by which mutations in the *NOD2* could be associated with the susceptibility to inflammation and development of diseases. When the exact mechanism of regulation and functions of *NOD2* will be unraveled, it could lead to the development of more effective therapies for inflammatory disorders.

## Disclosure

The authors report no conflicts of interest in this work.

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