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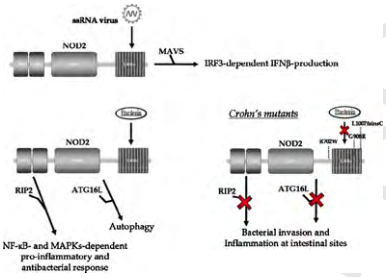
Graphical Abstract

The protein Nod2: An innate receptor more complex than previously assumed **pp: xxx–xxx**

Aurore Lecat, Jacques Piette, Sylvie Legrand-Poels*



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Review

The protein Nod2: An innate receptor more complex than previously assumed

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ABSTRACT

For almost 10 years, Nod2 has been known as a cytosolic innate receptor able to sense peptidoglycan from Gram-positive and -negative bacteria and to trigger RIP2- and NF-κB-mediated pro-inflammatory and antibacterial response. Mutations in the gene encoding Nod2 in humans have been associated with Crohn's disease (CD). Mechanisms by which Nod2 variants can lead to CD development are still under investigation. The most admitted hypothesis suggests that the impaired function of Nod2 variants in intestinal epithelial and phagocytic cells results in deficiencies in epithelial-barrier function which subsequently lead to increased bacterial invasion and inflammation at intestinal sites. Very recent results have just reinforced this hypothesis by demonstrating that Nod2 wild-type (unlike Nod2 variants) could mediate autophagy, allowing an efficient bacterial clearance and adaptative immune response. Other recent data have attributed new roles to Nod2. Indeed, Nod2 has been shown to activate antiviral innate immune responses involving IRF3-dependent IFN-β production after viral ssRNA recognition through a RIP2-independent mechanism requiring the mitochondrial adaptor protein MAVS. Recently, Nod2 has been also shown to be exquisitely tuned to detect mycobacterial infections and mount a protective immunity against these pathogens.

Contents

1. Introduction

In animals, the detection of infectious agents requires specific host pattern recognition receptors (PRRs) recognizing pathogen-associated molecular patterns (PAMPs). The identification and characterization of the membrane-bound receptors TLRs (Toll-like receptors) as PRRs have provided important insights in the mechanisms of host defense [1]. Ten years ago, most PRRs (including TLRs) were described as membrane receptors recognizing only extracellular PAMPs. By contrast, in plants, intracellular disease resistance (R) proteins involved in the host defense against specific pathogens have been already described [2].

Nod1 and Nod2 were identified in the early 2000s by Nùñez's team through homology to plant R proteins [3,4]. Indeed, both Nod and plant R proteins are characterized by a N-terminal effector domain, a centrally located nucleotide-binding domain (NBD) and multiple leucine-rich repeats (LRRs) in their C-terminal end. The N-terminal effector domains of Nod1 and Nod2 comprise one or two

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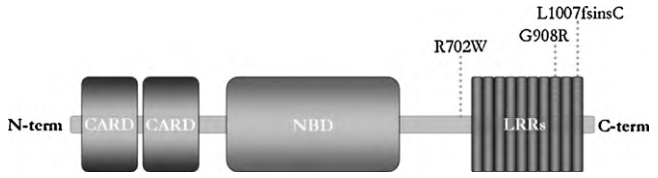

Fig. 1. Most common Nod2 mutants associated to Crohn's disease. Nod2 has a tripartite structure with two N-terminal CARD domains, a central NBD domain and C-terminal LRRs. The three most common polymorphisms associated with Crohn's disease are shown: two missense mutations R702W and G908R and a frameshift mutation L1007fsinsC.

caspase recruitment domains (CARD), respectively [3,4] (Fig. 1). While Nod1 is ubiquitous, Nod2 seems to be more restricted to myelomonocytic cells (monocytes/macrophages, granulocytes), dendritic cells and intestinal epithelial cells [4–6]. More importantly, Nod2 expression can be upregulated by pro-inflammatory stimuli in myelomonocytic and epithelial cells [5].

Very rapidly after their identification, Nod1 and Nod2 were shown to induce NF-κB activation after recognition of bacterial components through their LRRs and were suspected to play a role in innate immunity [7]. It was the first evidence showing the recognition of pathogen-associated compounds by cytosolic receptors in mammal cells. More interestingly, three variants within or near the LRRs domain of Nod2 were simultaneously identified and genetically associated with susceptibility to Crohn's disease (CD) [8,9] (Fig. 1).

Altogether, these data elicited many researches on the Nod2 protein concerning the Nod2-mediated transduction pathways, the precise bacterial structure recognized by Nod2, the mechanisms by which the Nod2 variants favor the CD and the physiologic roles of Nod2 in innate and adaptative immune response. The identification of Nod1 and Nod2 also stimulated homology searches revealing a growing family of structurally related proteins with centrally located NBD and carboxy-terminal LRRs. This family currently called NLR (Nod-like receptor) includes 23 members in humans and is divided in five subfamilies according to their effector domain [10]. Next to Nod1 and Nod2 known to induce the NF-κB signalling pathway, others NLR members such as NLRP1 and 3 (previously called NALP1 and 3) result in the activation of the inflammasome in response to specific PAMPs and DAMPs (danger-associated molecular patterns) [10]. It is now well admitted that the cytosolic NLR family contributes to the immune response against pathogens in collaboration with the membrane-bound TLRs and another recently identified family of cytosolic receptors called RLR (RIG-I-like receptor) [11].

This review is dedicated to the Nod2 protein and focuses on recent data attributing new potential roles to Nod2.

2. Nod2-mediated signal transduction pathways

2.1. Nod2-dependent NF-κB and MAPKs activation pathways in response to bacterial infection

The Nod-mediated NF-κB activation pathway has been extensively studied. Nod1 was initially identified by homology with Apaf-1, an inducer of apoptosis [3]. In addition of interacting with caspases, Nod1 was shown to interact with RICK (RIP-like interacting CLARP kinase), currently called RIP2 (receptor-interacting protein 2), a CARD-containing serine-threonine kinase and by this way to promote NF-κB activation [3]. The structural and functional similarity between Nod1 and Apaf-1 as well as those of RIP2 and RIP1, a serine-threonine kinase recruited to the TNFα receptor 1 (TNFR1) suggested a "induced proximity" model for RIP2 and RIP1-mediated NF-κB activation which is similar to the mechanism for activation of caspases during apoptosis [12]. According to this model, after Nod1 overexpression or TNFα binding, both Nod1 or TNFR1 could oligomerize and bind RIP2 or RIP1 by CARD homophilic or DD (death domain) interactions, respectively. RIP2 and RIP1 also bind to NEMO (NF-κB essential modifier) or IKKγ, the regulatory subunit of the IKK complex (IκBα kinase complex). The RIP2 or RIP1 recruitment to Nod1 or TNFR1 complexes, respectively, could allow the proximity of IKK complexes leading to NF-κB activation. Shortly after, Nod2 was identified and was shown to induce NF-κB activation by a similar RIP2-mediated mechanism [4]. In support of the "induced proximity" model, the kinase activity of RIP2 is not necessary for Nod1 and Nod2-mediated NF-κB activation but RIP2 seems to play simply the role of scaffold protein [12]. However, recent data highlighted the central role of the RIP2 kinase activity in conferring stability to the protein and accordingly in the preservation of Nod1 and Nod2-mediated innate immune responses [13].

Considering the structural homology between Nod1/Nod2 and plant R proteins, experiments were performed to determine whether Nod1/Nod2 might regulate the cellular response to microbial components. First, Nod1 and Nod2 were shown to confer responsiveness in terms of NF-κB activation to bacterial components through their LRRs [7]. However, when various bacterial sources were used, the Nod2 response pattern was shown to be different from that observed with Nod1. Indeed, after elucidation of the precise bacterial structure recognized by Nod1 and Nod2, it was clear that Nod1 and Nod2 were not devoted to sense the same pathogens. While the minimal motif detected by Nod1 is the peptidoglycan (PGN) fragment γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP), Nod2 recognizes the PGN fragment MurNAc-L-Ala-D-isoGln called MDP for muramyl dipeptide [14,15]. The iE-DAP is commonly present in Gram-negative bacteria whereas MDP is found in both Gram-negative and Gram-positive bacteria (Fig. 2). The mechanism by which Nod1 and Nod2 sense iE-DAP or MDP, respectively, is currently unknown. It probably involves not yet identified partners.

The identification of MDP or iE-DAP as Nod-activating PAMPs elicited questions. For example, how a subunit of PGN can reach a cytosolic receptor? Several mechanisms were proposed according to the cell type and the bacterial agent. Fig. 3 summarizes what is known about the modes of MDP intracellular delivery. The first mechanism concerns invasive bacteria which, after their internalization in non-phagocytic cells, can escape from the vacuole into the cytosol where they ~~replicate~~ [16]. PGN must be continuously remodeled during bacterial growth. In some Gram-negative bacteria, PGN breakdown mediated by transglycosylases and peptidases releases fragments in the periplasm which are re-imported into the cytoplasm for recycling [17]. A minor fraction of these PGN fragments can be exported in the cytosol of infected mammal cells (Fig. 3A). Indeed, it was shown that muramylpeptides shed by *Shigella* in epithelial cells are able to trigger Nod1-mediated NF-κB activation [18]. Muramylpeptides can be also generated in the cytosol after invasion by Gram-positive bacteria. Indeed, the Nod2 ligand, muramyl dipeptide (MDP) can potentially be generated in *Listeria monocytogenes*-infected cells upon PGN cleavage by secreted autolysins p60 and NamA [19] (Fig. 3A). Bacterial components can be also delivered into the cytosol through bacterial secretion systems or pore-forming toxins. While this mode of intracellular delivery allows PAMPs detection by some members of the NLR family, such a report was not yet described for Nod2. A third mechanism allowing PAMPs release inside cytosol involves phagocytosis by macrophages. Nod2 was shown to be activated in stimulated macrophages by bacterial ligands generated in the phagosome and transported to the cytosol [20] (Fig. 3B). Finally, extracellular MDP can get access to the host cytosol through the intestinal apical di-/tripeptide transporter,

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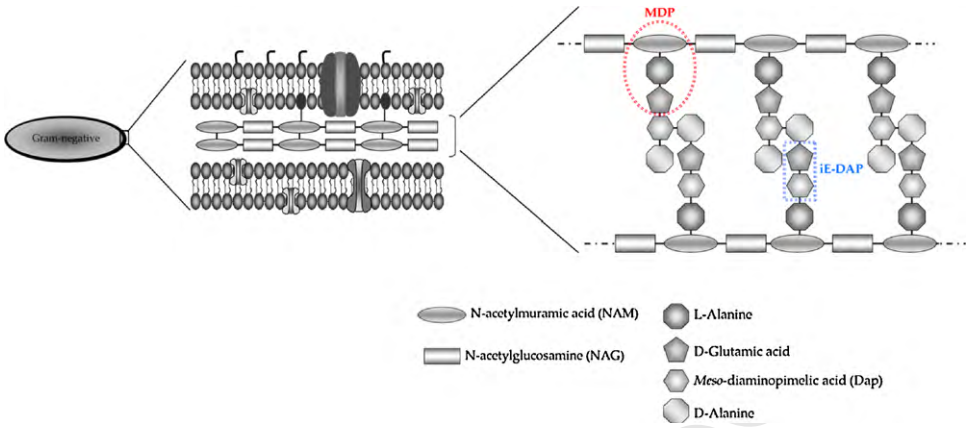

Fig. 2. Nod1 and Nod2 agonists. A schematic representation of Gram-negative bacteria cell wall is shown with the periplasmic space comprised between outer and inner membranes. Peptidoglycan located in the periplasm is composed of linear chains containing two alternating amino sugars, N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM) attached by short peptides. The minimal motif recognized by Nod1 is the fragment γ-D-glutamyl-meso-diaminopimelic acid (iEDAP) while Nod2 detects the PGN fragment MurNAc-L-Ala-D-isoGln called MDP for muramyl dipeptide.

hPepT1 [21] or by a ~~clathrin-and~~ dynamin-dependent endocytic pathway [22,23] (Fig. 3C).

The subcellular distribution of Nod2 was also further investigated. Besides its cytosolic location, Nod2 is associated with the plasma membrane in epithelial and monocytic cells [24–29]. Interestingly, Nod2 localizes to the basolateral membrane in polarized intestinal epithelial cells [25]. Nod2 as well as Nod1 are also recruited in membrane ruffles through Rac1 interaction and the NF-κB activation pathways mediated by Nod2 and Nod1 are modulated by actin cytoskeleton [27,29,30]. In MDP-treated monocytic cells, Nod2 is translocated to the membrane by a Rac1-dependent mechanism [29]. By the same way, Nod2 and

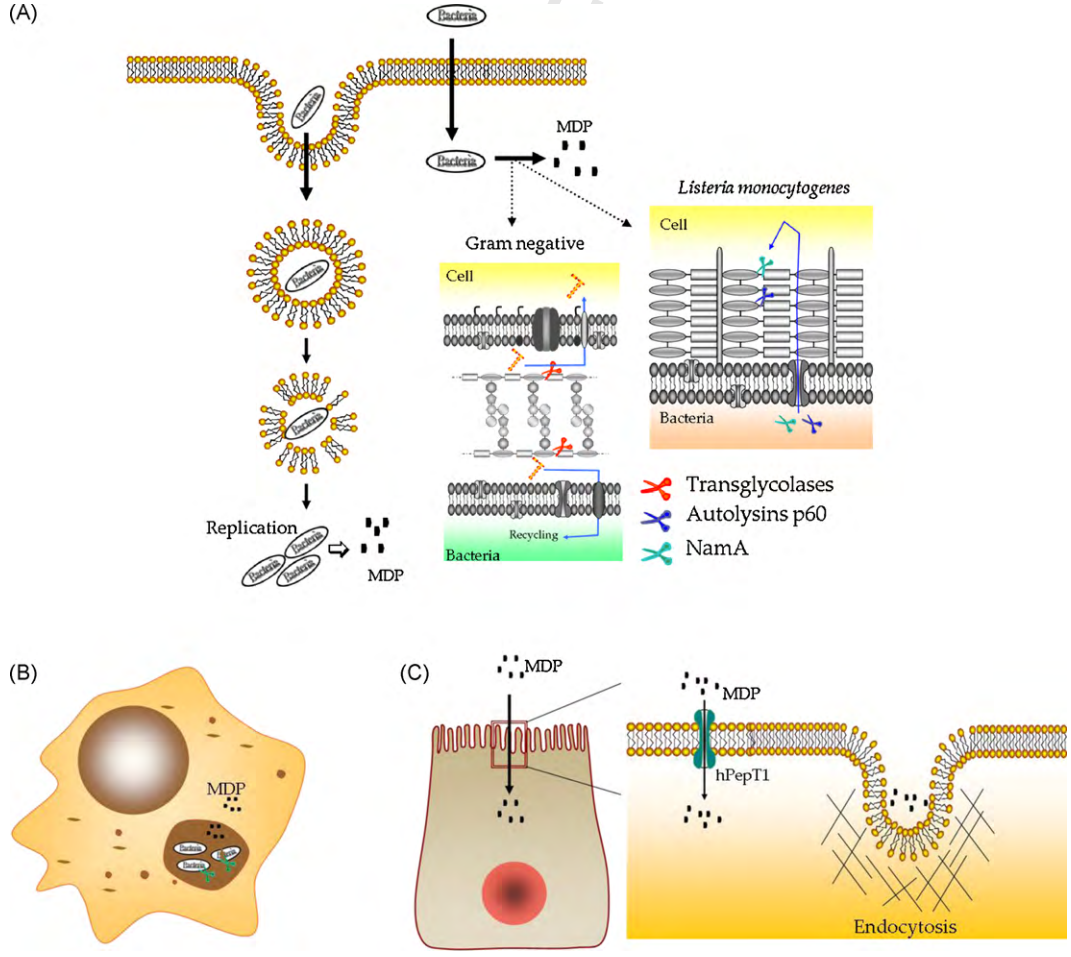

Fig. 3. Modes of MDP intracellular delivery. (A) MDP intracellular delivery by invasive bacteria. Muramylpeptides are shed by Gram-negative bacteria or released after *L. monocytogenes* PGN cleavage by secreted autolysins. (B) MDP intracellular release during phagocytosis. (C) Extracellular MDP transport into the cell through the intestinal transporter hPepT1 or endocytosis.

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Nod1 are recruited to invasion foci of *Shigella flexneri* in HeLa cells, probably also by a mechanism dependent on actin cytoskeleton [26,30]. The recruitment of Nod2 in Rac1-induced dynamic cytoskeletal structures could be a strategy to rapidly mobilize Nod2 during MDP endocytosis or bacterial invasion and to ensure a close proximity between Nod2 and its ligand. Furthermore, Nod2 promotes the membrane recruitment of RIP2, the serine–threonine kinase involved in NF-κB activation downstream of Nod2 [28]. This membrane compartmentalization mechanism seems to be required for an optimal activity of the Nod2-RIP2 complexes and has been already described for integral membrane TLRs and TNFRI to regulate downstream signalling [31,32]. Among the three most common mutants associated with CD, the $Nod2^{1007FS}$ mutant, deleted from its 33 carboxy-terminal amino acid residues, is the only one to be unresponsive to MDP and to be delocalized in the cytoplasm [24,28]. However, the targeting of the $Nod2^{1007FS}$ mutant to the plasma membrane did not restore the ability to activate NF-κB in response to MDP meaning that this variant has another defect making it unable to recognize MDP [28].

Since 2003, several non-degradative ubiquitination events have been shown to play a key role in various NF-κB activation pathways [33]. The MDP-induced Nod2 signalling also appeared to involve ubiquitination steps. Indeed, Nod2 was shown to induce RIP2-dependent K63-linked ubiquitination of NEMO at K285, and NEMO ubiquitination was impaired by Nod2 mutations associated with Crohn's disease [34]. Subsequent studies revealed that Nod2-mediated NF-κB activation requires K63-linked ubiquitination of RIP2; the key ubiquitination acceptor site was mapped to K209 [35]. Both polyubiquitination events promote the recruitment of the TAK1 kinase, which activates IKK complex [35–38]. Very recently, cIAP1 and cIAP2 (cellular inhibitor of apoptosis proteins 1 or 2) were identified as E3 ubiquitin ligases required for RIP2 ubiquitination and Nod2-mediated NF-κB activation [39].

The current model of Nod2-mediated NF-κB activation is illustrated in Fig. 4. In resting cells, Nod2 stays in an autoinhibited conformation through intramolecular inhibition of the NBD domain by LRRs [4]. Upon MDP recognition through LRRs, conformational modifications allow nucleotide triphosphate binding and Nod2 oligomerization through the NBD. MDP or bacterial invasion also promote Nod2 recruitment to the plasma membrane or invasion foci, respectively, by a cytoskeleton-dependent mechanism [26,29]. Nod2 oligomerization induces the RIP2 recruitment through homotypic CARD-CARD interactions. RIP2 interacts with IKKγ through its intermediate region. The induced proximity of RIP2 molecules promotes the K63-linked polyubiquitination of NEMO as well as its own K63-linked polyubiquitination achieved by cIAP1 and cIAP2 at lysine 209 located in its kinase domain. Both ubiquitination events are crucial for the TAK1 complex recruitment allowing the subsequent IKK complex activation through the phosphorylation of the IKKβ subunit. This is followed by the phosphorylation and degradation of IκBα, releasing NF-κB which can then translocate to the nucleus and transactivate targets genes. Interestingly, Nod2-mediated NF-κB activation proceeds by a mechanism very similar to the one used by TNFR1 [33]. Indeed, after activation by their respective ligand and oligomerization, TNFR1 or Nod2 recruit RIP1 or RIP2 to the plasma membrane, respectively. Both scaffold proteins RIP1 or RIP2 interact with NEMO and become K63-linked polyubiquitinated by cIAP1 and cIAP2. These events are followed by TAK1 complex recruitment and IKK complex activation.

MDP recognition by Nod2 also leads to the activation of MAPKs (mitogen-activated protein kinases), including p38, ERK (extracellular signal-regulated protein kinase) and JNK (c-Jun N-terminal kinase) [37]. Although the scaffold protein RIP2 and the kinase TAK1 are also involved in the Nod2-mediated MAPKs activation (Fig. 4), the downstream effectors are less well characterized. Little is known about the molecular mechanisms by which Nod2 functionally coordinates separate signalling pathways. Some signalling intermediates have been shown to differentially modulate the Nod2-mediated NF-κB and MAPKs activation. It is the case of the adaptor protein CARD9 which associates with Nod2 in response to MDP and has a critical function in Nod2-dependent p38 and JNK activation but no effect on Nod2-induced NF-κB

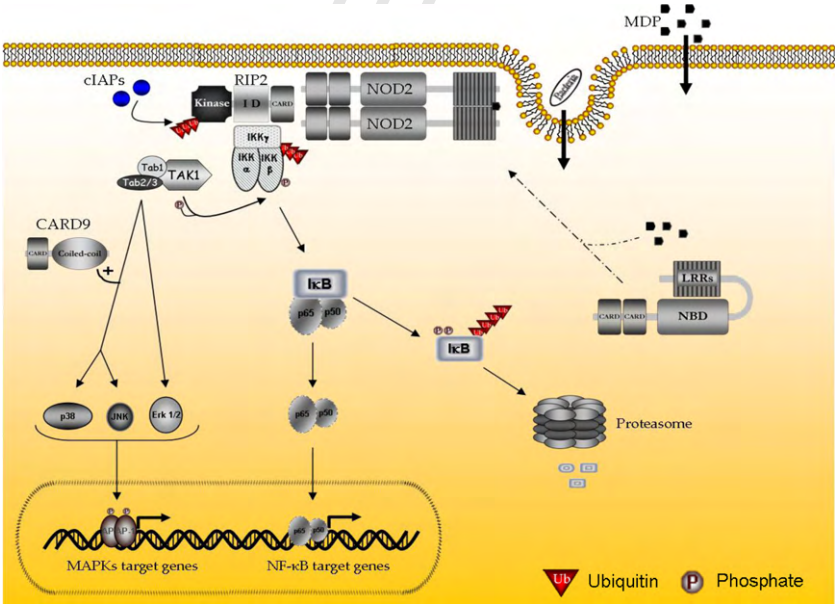

Fig. 4. Nod2-mediated NF-κB and MAPKs activation ~~pathway~~ in response to bacterial infection. In resting cells, Nod2 stays in an autoinhibited conformation through intramolecular inhibition of the NBD domain by LRRs. Upon MDP recognition through LRRs, conformational modifications allow nucleotide triphosphate binding and Nod2 oligomerization through the NBD followed by RIP2 recruitment through homotypic CARD-CARD interactions. RIP2 also interacts with IKKγ through its intermediate region. The induced proximity of RIP2 molecules promotes the K63-linked polyubiquitination of IKKγ as well as its own K63-linked polyubiquitination achieved by cIAP1 and cIAP2 at lysine 209 located in its kinase domain. Both ubiquitination events are crucial for the TAK1 complex recruitment allowing the subsequent IKK complex activation through the phosphorylation of the IKKβ subunit. This is followed by the phosphorylation and degradation of IκBα, releasing NF-κB which can then translocate to the nucleus and transactivate target genes. RIP2 and TAK1 recruitment also induce MAPKs activation. ~~CARD 9~~ plays a critical role in Nod2-mediated p38 and JNK activation.

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pathway [40]. MEKK4, a MAP3K which binds to RIP2, is required for Nod2-induced p38 and ERK activation while it downregulates Nod2-dependent NF-κB pathway [41]. Another report has shown that the K63-linked polyubiquitination of RIP2 on another residue as K209 by the E3 ubiquitin ligase ITCH primes the Nod2-mediated MAPKs pathway at the expense to the NF-κB one [42].

Both NF-κB and MAPKs pathways contribute to the Nod2-mediated inflammatory and immune response. In vitro studies have shown that activation of human epithelial cells by MDP induces the production of pro-inflammatory cytokines (TNFα, IL-6), chemokines (IL-8, MIP2, MCP-1,...) and antimicrobial peptides [43]. In antigen-presenting cells, activation of Nod2 also leads to the secretion of pro-inflammatory cytokines such as IL-6, TNFα, IL-1β, IL-10, IL-18, IL-12 and chemokines IL-8 and RANTES [43,44].

Several proteins exerting modulating functions on Nod2-mediated NF-κB activation were identified in the search for binding partners of Nod2. The cell polarity protein Erbin, the GTPase-activating protein Centaurin-β1 (CENTB1) and the angio-associated migratory cell protein (AAMP) bind to Nod2 and negatively regulate Nod2-dependent NF-κB activation while the GRIM-19 protein that also associates with Nod2 appears to positively modulate Nod2 signalling [25,26,45–47]. Since Erbin and CENTB1 expression is upregulated after Nod2 activation, both proteins could mediate a negative feedback.

2.2. Nod2-mediated IRF3 activation in response to viral infection

Very recent results have expanded the function of Nod2 beyond detection of peptidoglycan [48]. Indeed, Sabbah et al. found that Nod2 (and not Nod1) could confer responsiveness to ssRNA in terms of IRF3 activation and IFN-β production. Furthermore, wild-type cells expressing Nod2-specific small interfering RNA or BMMs (bone marrow-derived macrophages) from Nod2-deficient mice failed to induce an antiviral response after ssRNA transfection. A significant Nod2-mediated IRF3 activation was also observed after infection with RSV (respiratory syncytial virus), VSV (vesicular stomatitis virus) or influenza virus, each of them has a ssRNA genome.

Like with MDP, the ssRNA recognition mechanism is still unknown. The authors demonstrated the recovery of VSV-specific RNA in Nod2-containing complexes after VSV infection which does not likely result from a direct binding between Nod2 and ssRNA but rather involves bridging proteins.

To further investigate the molecular mechanism involved in Nod2-dependent IRF3 activation, authors turned to MAVS, the mitochondrial antiviral signalling protein which mediates RLRs (RIG-I and MDA-5)-induced antiviral response [49]. They showed that MAVS extinction impaired Nod2-dependent IFN-β production after RSV infection. Interestingly, after RSV infection, Nod2 translocated to the mitochondria where it associated with MAVS (Fig. 5). Unlike RIG-I, the CARDs of Nod2 were not important for its interaction with MAVS. However, the NBD and LRR domains were required for the MAVS association and Nod2-dependent IRF3 activation. Interestingly, another NLR family member, NLRX1, was shown to interact with MAVS through its NBD and LRR domains leading to a downregulation of RIG-I- and MDA5-mediated antiviral response [50].

Since the expression of interferon genes also requires NF-κB activation in addition to IRF3, Sabbah et al. confirmed it was also the case for Nod2-mediated IFN-β production. On the basis of these results, they speculated that the mechanism by which Nod2 induces IFN-β production in response to ssRNA is similar to that of RLRs and involves the mitochondrial adaptor MAVS which, in turn, recruits downstream signalling complexes for NF-κB and IRF3 activation [49].

Altogether, these data demonstrate that Nod2 can trigger at least two distinct pathways from two different cellular locations

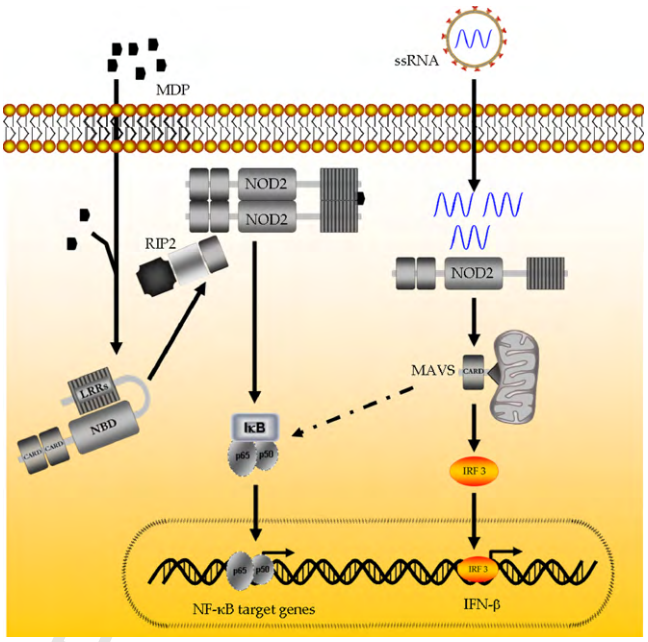

Fig. 5. Two distinct Nod2-mediated pathways: MDP-induced pro-inflammatory and antibacterial pathway initiated from the plasma membrane and ssRNA-induced antiviral pathway originating from the mitochondria. MDP recognition by Nod2 leads to NF-κB and MAPKS activation through a RIP2-mediated mechanism while ssRNA sensing by Nod2 induces IRF3 activation through MAVS.

(Fig. 5): the MDP-induced pro-inflammatory and antibacterial pathway initiated from the plasma membrane after recruitment of RIP2 and downstream signalling complexes leading to NF-κB and MAPKs activation and the ssRNA-induced antiviral pathway originating from the mitochondria after Nod2 association with MAVS and recruitment of the downstream signalling complexes required for NF-κB and IRF3 activation. Interestingly, transfection of Nod2 alone can induce the RIP2-dependent pathway without any external stimuli [4] but cannot trigger IRF3 activation in the absence of viral ssRNA [48]. This observation suggests that Nod2 involvement in each pathway is fundamentally different. All these new data are also very well summarized and illustrated in a recent review on NonInflammasome NLRs [51].

2.3. Nod2-mediated autophagy in response to bacterial infection

Autophagy is a highly conserved degradation process in which portions of cytoplasm or damaged organelles become surrounded by a double-membrane delineating a vacuole called autophagosome which is eventually targeted for fusion with lysosomes [52]. This mechanism usually cytoprotective is induced in response to many stress conditions [53]. In particular, autophagy has emerged as a critical pathway of host defense against viral, bacterial and parasitic infections [54]. A link between intracellular bacteria-sensing receptors and the induction of autophagy has already been established in plants and insects [55,56]. Such a link between the NLR receptors, Nod1 and Nod2, and autophagy has just been demonstrated in mammals [57,58].

Travassos et al. showed that the intracellular delivery of iEDAP or MDP induces autophagy by a mechanism dependent on Nod1 or Nod2, respectively [57]. Accordingly, the autophagy triggered by invasive bacteria known to be sensed by Nod1 or Nod2 was shown to require Nod1 or Nod2, respectively, and contribute to Nod1 or Nod2-mediated host defense [57,58]. Both Nod1 and Nod2-induced autophagy by a mechanism independent on NF-κB but involving the key autophagy regulator ATG16L1 [57] (Fig. 6). Both Nod proteins interacted and colocalized with ATG16L1 at the

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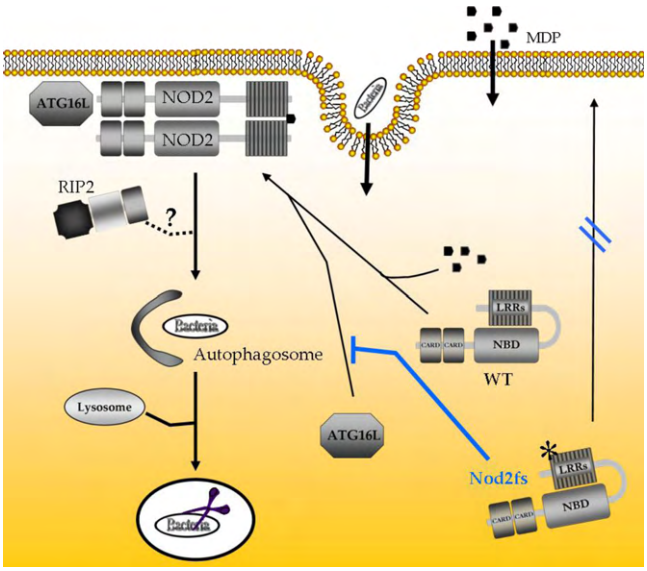

Fig. 6. Nod2-mediated autophagy in response to bacterial invasion. Nod2 can serve as a molecular scaffold for the autophagy machinery. The most common variant Nod2L1007fsinsC associated to Crohn's disease, by sequestering ATG16L1, fails to induce autophagy.

plasma membrane. During bacterial invasion, ATG16L1 was recruited to bacterial entry sites with Nod1 or Nod2. Their findings suggest that Nod proteins serve as a molecular scaffold for the autophagy machinery and by this way act as nucleation sites for autophagy initiation [57]. RIP2 involvement in Nod2-dependent autophagy is a matter of debate. Indeed, Travassos et al. showed Nod2-dependent autophagy was not impaired in RIP2-deficient MEFs, while Cooney et al. demonstrated a role of RIP2 in Nod2-mediated autophagy in human dendritic cells (DCs) through RIP2-specific siRNA transfection or RIP2 inhibitor treatment [57,58]. It will be crucial to define the exact molecular complex by which Nod2 acts to induce autophagy.

As previously reported, the most common CD mutant, Nod2fs, failed to localize to the plasma membrane [24]. Interestingly, Travassos et al. also showed that Nod2fs sequesters ATG16L1 in the cytosol and thereby prevents it from translocating to bacterial invasion foci and initiating autophagy (Fig. 6) [57].

3. Nod2 involvement in other signalling pathways

3.1. Antiviral pathway involving OAS2-RNase-L

The expression of oligoadenylate synthetases (OAS) is induced in response to type I interferons [59]. These enzymes are activated by viral dsRNA binding. OAS-dsRNA complexes are then able to convert ATP into 2′,5′-linked oligomers of adenosine (2-5A) which, in turn, can bind to RNase-L, thereby enabling RNase-L to cleave viral and cellular RNAs. Both degradation events impair further viral production. On the other hand, by cleaving some self mRNAs, RNase-L produces small RNAs which act as a ligand for intracellular receptors such as RIG-I and MDA-5. These receptors mediate a positive feedback phenomenon by inducing type I IFNs production.

Nod2 was shown to interact with OAS2 and enhance RNAse-L activity in cells treated with poly(I:C), a mimic of double-stranded RNA virus infection [60]. These results suggest that Nod2 would be not only able to mediate IFN-β production after infection by ssRNA virus but could be also involved through an indirect mechanism in RLRs-dependent type1 interferons expression after dsRNA virus infection. This last point needs to be further explored.

3.2. Antibacterial pathway involving DUOX2-induced ROS production

Given the link between plant R proteins and pathogen-induced reactive oxygen species (ROS) production [61] as well as the interaction between NOX family members and components of the TLR signalling cascade [62], Lipinski et al. explored whether Nod2 signalling mediated ROS production and if this mechanism contributed to host defense [63]. They showed a rapid and significant Nod2-dependent ROS production in MDP-stimulated intestinal epithelial cells (IECs) which was mainly mediated by DUOX2, a dual oxidase generating hydrogen peroxide. Interestingly, DUOX2 expression was upregulated by inflammatory stimuli and was increased in IECs originating from inflamed tissue in comparison to non-inflamed tissue. Nod2 was shown to interact with DUOX2 through its LRRs. Nod2 mediated a DUOX2-dependent protection against bacterial cytoinvasion which could be attributed to a direct effect involving the generation of bactericidal ROS and an indirect mechanism consisting of NF-κB signalling upregulation by ROS. Interestingly, the Nod2L1007fsinsC mutant did not lead to a significant ROS production and gave only reduced protection against bacterial cytoinvasion.

4. Role of Nod2 in innate immune response

The identification of the Nod proteins sensing the same pathogens and triggering the same transduction pathways as TLRs elicited the question of redundancy between these PRRs. Several studies in vitro demonstrated that Nod2 and Nod1 agonists have a synergistic effect on TLRs-mediated inflammatory cytokines production, which could potentiate the cellular response against pathogens [64–66]. A role of Nod2 as a negative regulator of TLR2-mediated IL-12 secretion has also been suggested but does not seem to be a universal phenomenon [67,68]. On the other hand, an essential role of Nod2 in host defense against some pathogens has been demonstrated in some in vivo models.

Nod2$^{-/-}$ mice challenged via intragastric dosing with *L. monocytogenes*, a Gram-positive enteroinvasive bacteria, were more susceptible to infection than wild-type mice [68]. However, Nod2$^{-/-}$ mice challenged with *L. monocytogenes* by intravenous or intraperitoneal injection did not show any significant difference neither in bacteria recovering nor in survival. These data emphasize the critical role of Nod2 in innate immunity in the intestinal tract. Nod2 could play this role at least partially by mediating the expression of intestinal antimicrobial peptides known as cryptdins in mice and α-defensins in humans [68]. Furthermore, in mice made insensitive to TLRs by previous exposure to microbial ligands and then submitted to systemic infection with *L. monocytogenes*, Nod1 and Nod2 were required for the bacterial clearance and mice survival [69]. Altogether, these data suggest that Nod2 plays a critical role in host defense when TLRs signalling is reduced like in intestinal cells or inhibited via tolerization. Such a tolerization phenomenon can originate from the continuous exposition to commensal bacteria like it is the case for intestinal cells.

Several studies also focused on the potential involvement of Nod2 in protective immunity to other intracellular pathogens such as mycobacteria. In general, the secretion of pro-inflammatory cytokines by monocytes/macrophages in response to infection by Gram-positive or Gram-negative intracellular bacteria is not significantly affected by Nod2 deficiency because TLRs often well-expressed in this kind of cells fulfill this function. By contrast, Nod2 was required for optimal inflammatory response in macrophages and dendritic cells after infection with live *Mycobacterium tuberculosis*, suggesting Nod2 and TLRs are non-redundant recognition systems of *M. tuberculosis* [70–72]. Furthermore, in *M. tuberculosis*-infected mouse model, Nod2 deficiency ultimately

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led to higher pulmonary bacterial burden and decreased host resistance to chronic mycobacterial infection [73]. The same authors further investigated the mechanism by which Nod2 mediates a so important contribution in innate immune response against *M. tuberculosis* in comparison with other intracellular bacteria. An interesting obervation is that mycobacteria convert their MDP into an N-glycolylated form through the action of N-acetyl muramic acid hydroxylase (NamH) while MDP is N-acetylated in most bacteria [74]. They showed that N-glycolyl MDP is more potent than N-acetyl MDP in activating Nod2-mediated signalling in vitro [75]. In mice challenged intraperitoneally with live wild-type *Mycobacterium smegmatis* or *nam*H-deficient *M. smegmatis*, Nod2-dependent immune responses were disrupted in the absence of NamH [75]. Altogether, these data suggest that Nod2 may be exquisitely tuned to detect mycobacterial infections.

Very recently, Nod2 was also shown to be a critical component of host antiviral defense mechanisms. Indeed, after having shown that viral ssRNA or RSV infection induced Nod2-mediated IFNβ production in vitro, Sabbah et al. infected wild-type or Nod2-deficient mice with a sublethal dose of RSV by intranasal inoculation [48]. They observed an increase of Nod2 expression in RSV-infected lungs which correlated with the interferon-induction kinetics, suggesting an important role for Nod2 in interferon production. Indeed, Nod2-deficient mice had lower IFN-β production in the respiratory tract and higher viral titers than wild-type mice. Nod2$^{-/-}$ mice also showed more severe lung pathology, lost considerably more body weight and had diminished survival relative to their wild-type counterpart.

5. Role of Nod2 in adaptative immune response

The innate immune system not only represents the first line of defense against invading pathogens but it is also responsible for instructing appropriate adaptative immune responses. While the key role of Nod2 protein in innate immune defense has been established, until recently few studies have examined its contribution to the adaptative immune response. However, MDP had already been identified as the minimum effective component of CFA (complete Freund's adjuvant) in 1974 [76]. It is only in 2005, following the identification of MDP as a Nod2 agonist [14,15], that its adjuvanticity could be attributed to Nod2, since Nod2-deficient mice could not mount a normal humoral response after immunization with MDP plus an antigen [68]. Since then, the mechanism by which MDP exerts its adjuvant activity through Nod2 was further explored.

MDP-induced Nod2 signalling in human dendritic cells (DCs) was shown to promote the differentiation of human memory CD4$^+$ T cells into T-helper 17 (Th17) cells [77]. In a mouse model, MDP-stimulated Nod2 triggered a potent Ag-specific immune response with a Th2-type polarization profile [78]. In this same model, Nod2 was also critical for the induction of both Th1- and Th2-type responses following costimulation with TLR agonists. Dendritic cells were shown to play a central role in this synergistic priming of adaptative immunity.

TLRs are known to influence the adaptative immune response not only by inducing the expression of costimulatory molecules but also by priming the antigen presentation pathway in dendritic cells, both events being required for naïve T cells activation [79]. Since Nod2 has been recently shown to mediate autophagy [58] and given that autophagy has a major role in antigen presentation, with constitutive fusion of autophagosomes with multivesicular MHC class II-loading compartments in antigen-presenting cells [80], the potential link between Nod2 and MHC class II antigen presentation machinery was investigated. MDP treatment of primary immature human DCs induced Nod2-dependent autophagy, which, in turn, led to an increase of MHC class II (but not of MHC class I) surface levels [58]. To further determine the effect of Nod2-mediated autophagy on antigen presentation, Cooney et al. infected DCs with *Salmonella enterica* or recombinant *S. enterica* Cfr expressing the C-fragment of tetanus toxin before exposure to autologous CD4$^+$ T cells of tetanus-immune individuals. The antigen-specific proliferation of autologous CD4$^+$ T cells was significantly reduced after knockdown of Nod2 or ATG16L1, a key autophagy mediator, suggesting that Nod2-dependent autophagy can contribute to MHC class II antigen presentation (Fig. 7A). Consistent with previous data showing that the CD mutant Nod2fs failed to induce autophagy, Nod2fs-expressing DCs, after infection with *S. enterica* Cfr, were much less efficient to stimulate tetanus toxin-specific CD4$^+$ T cells proliferative responses.

In another very recent work, Nod signalling was shown to enhance DCs-mediated cross-priming [81]. DCs have the unique ability to deliver exogenous Ag to the MHC class I-restricted Ag presentation pathway and generate CD8$^+$ T cell immunity to viral infection and cancer, a phenomenon known as cross-priming [82]. In this study, authors demonstrated that injecting ligands for Nod2 (and Nod1) along with Ag (OVA) into wild-type mice significantly enhanced the cross-priming of OVA-specific CD8$^+$ T cells by CD8α$^+$ DCs. Cross-presentation can proceed according to several mechanisms [82]. OVA cross-presentation is mediated by the early endosomes pathway. In this model, soluble antigens taken up by the mannose receptor (MR) and targeted to a stable early endosomal compartment are translocated into the cytosol where they are degraded into antigenic peptides by the proteasome (Fig. 7B). These peptides are targeted again to early endosomes through the TAP protein before being loaded onto MHC class I molecules for presentation at the cell surface (Fig. 7B). The authors demonstrated that injecting Nod2 (or Nod1) ligands with OVA into

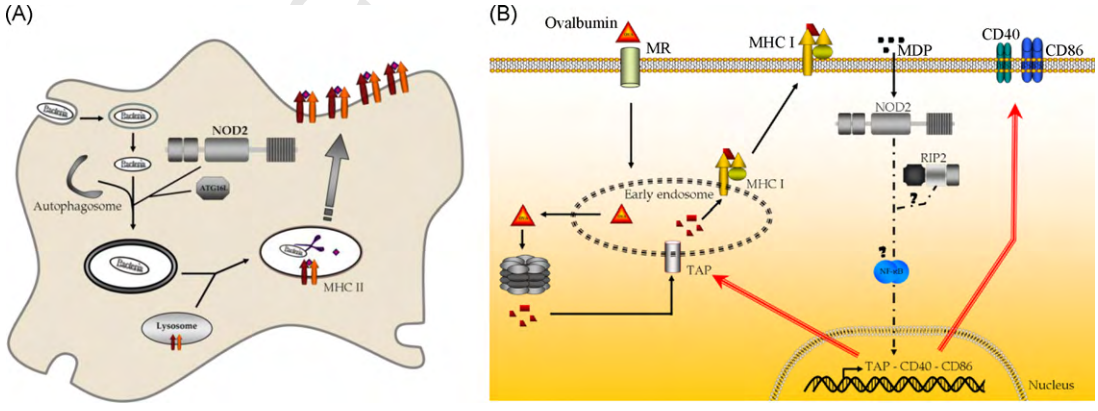

Fig. 7. Effects of Nod2 on MHC class II antigen presentation and cross-presentation by dendritic cells. (A) Nod2-dependent autophagy can mediate MHC class II antigen presentation. (B) Upregulation of the cross-presentation and costimulatory molecules by Nod2 signalling.

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wild-type mice upregulated DCs expression of molecules involved in cross-presentation such as Tap-1, calnexin,... Furthermore, the expression of surface costimulatory molecules (CD40, CD86) was also increased. Accordingly, they postulated that Nod signalling enhanced the cross-priming of Ag-specific $CD8^+$ T cells by $CD8\alpha^+$ DCs, at least in part, by upregulating Ag cross-presentation pathway and costimulation via surface molecules (Fig. 7B). However, while the authors checked that Nod1 ligand failed to enhance the cross-priming in Nod1-deficient mice, they did not perform the same experiment with $Nod2^{-/-}$ mice. Unfortunately, they did not investigate neither the mechanism by which Nod stimulation led to increased expression of cross-presentation or costimulatory molecules, the involvement of RIP2, NF-κB and MAPKs nor the behaviour of CD mutants. Nevertheless, these preliminary results might open onto the development of novel preventive and therapeutic applications for viral infections as well as cancer.

Finally, another recent study challenged the current dogma that Nod2 regulates adaptative immunity through MDP sensing in DCs by showing a T cell-intrinsic role of Nod2 independent on MDP recognition [83]. Authors demonstrated that $Nod2^{-/-}$ $CD4^+$ T cells had poor helper T cell differentiation, which was associated with impaired production of IL-2. Preliminary results suggested a scaffold role for Nod2 in the pathway leading to c-Rel-mediated IL-2 transcription in response to CD28 costimulation. These findings are conflicting with previous studies showing that Nod2 is not required in generating effective Ag-specific responses after immunization with other agonists than MDP [68,78] or with data demonstrating no T cell-intrinsic role for Nod2 [84]. This intringing study will likely generate further research on Nod2 behaviour in T cells.

6. Nod2 and ~~crohn~~ disease

At least 58 disease-associated mutations in *nod2* gene (CARD15) have been reported [85]. Approximately 80% of these are associated with Crohn's disease (CD), while the remaining 20% have been linked with Blau syndrome (BS) and early onset sarcoidosis (EOS).

Most patients with BS/EOS have mutations affecting the Nod2 NBD domain which lead to increased basal and MDP-induced NF-κB activity [86]. BS/EOS is usually considered as an autoinflammatory disorder characterized by the usual triad of granulomatous arthritis, uveitis and dermatitis which develop very early (before 4 years) [85].

CD is a multifactorial inflammatory bowel disease (IBD). Unlike BS/EOS which typically have diffuse, multisystemic granulomatous inflammation, CD is characterized by transmural granulomatous inflammation of the gastrointestinal tract. Three major Nod2 polymorphisms are associated to CD: G908R, R702W and a frameshift insertion mutation at L1007 (L1007fsinsC) [8,9]. Patients homozygous for these mutations have a 20- to 40-fold increased risk for disease development.

Since these mutations are localized within or near the LRR domain of Nod2, they prevent CD variants to be efficiently activated by MDP and consequently lead to a loss-of-function phenotype [14]. PBMCs isolated from CD patients carrying the L1007fsinsC mutation express reduced levels of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-8 in response to MDP [87,88]. However, these observations are inconsistent with the increase of NF-κB-dependent inflammation observed in clinical samples from CD patients. Two major hypotheses establish a link between these "loss-of-function" CD mutants and the development of CD.

The first hypothesis suggests that the impaired function of Nod2 in intestinal epithelial and phagocytic cells results in deficiencies in epithelial-barrier function which subsequently lead to increased bacterial invasion and inflammation at intestinal sites (Fig. 8). Several Nod2-regulated phenomenons can contribute to the impairment of epithelial-barrier function. On one hand, the Nod2-dependent production of antimicrobial peptides such as

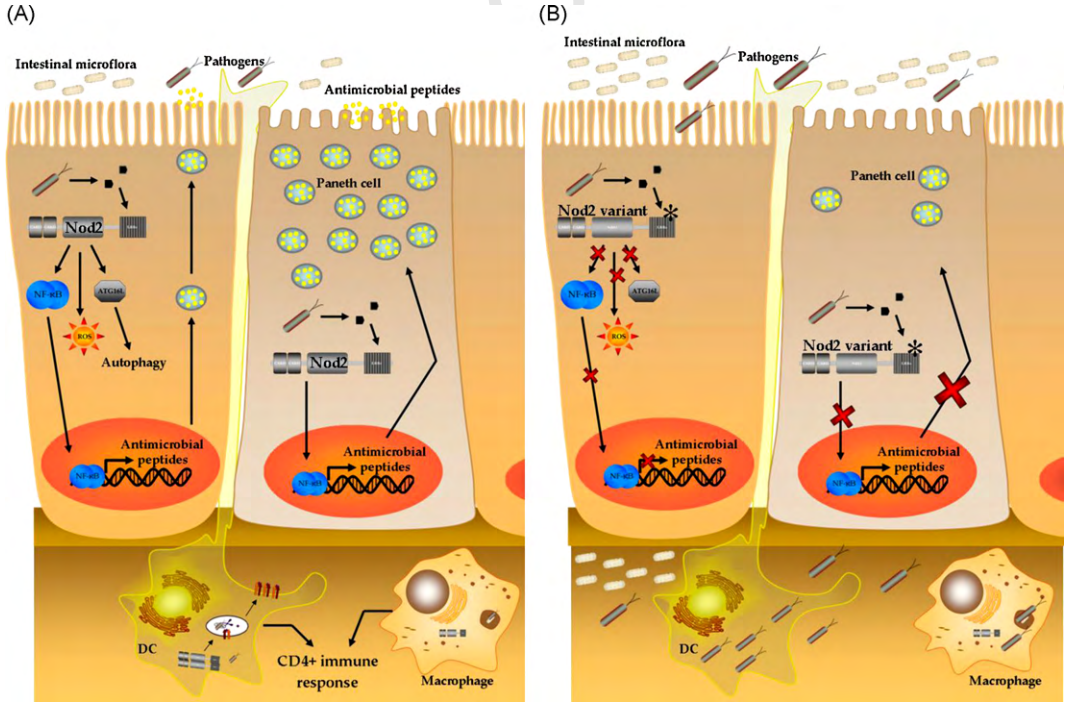

Fig. 8. Nod2-dependent alterations that affect the intestinal epithelium-barrier function in Crohn's disease. (A) Normal intestinal epithelium. Paneth cells respond to pathogens and commensal bacteria by secreting antimicrobial peptides. Epithelial cells, local macrophages and dendritic cells contribute to the bacterial clearance by mounting an efficient autophagy response and/or bactericidal ROS production. These innate protection mechanisms associated with an efficient $CD4^+$ T immune response cooperate in the control of bacterial invasion. (B) Intestinal epithelium expressing Nod2 variant. Antimicrobial peptides secretion, ROS generation and autophagy do no longer work. These alterations in innate response in combination with an inadequate $CD4^+$ T immune response promote the bacterial persistence and the generation of secondary inflammatory changes characteristic of CD.

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α-defensins can be downregulated in Paneth cells expressing CD mutant, which can lead to overgrowth of the intestinal microflora and increased susceptibility to enteric pathogens. This hypothesis is consistent with two observations: first, the reduced expression of α-defensins at ileal mucosa of CD patients with mutant Nod2 [89] and secondly, the correlation between the increased susceptibility of Nod2-deficient mice to *L. monocytogenes* oral infection and diminished expression of Paneth cell-derived antimicrobial peptides [68]. On the other hand, the Nod2-dependent bacteria clearance mechanisms such as autophagy and bactericidal ROS production can be compromised in intestinal epithelial and dendritic cells expressing a CD-associated Nod2 mutant [57,58,63]. Interestingly, a "loss-of-function" mutation (T300A) in ATG16L1, a key regulator of autophagy, was also associated with a higher susceptibility for CD [90]. The findings demonstrating that two of the strongest genetic risk factors in CD (Nod2 and ATG16L1) are linked in the autophagy pathway underline the important role of autophagy in intestinal homeostasis. The inability of DCs expressing one or both variants (Nod2 L1007fsinsC or ATG16L1 T300A) to traffic bacteria and present antigens correctly could lead to inadequate generation of $CD4^+$ T immune responses, facilitating bacterial persistence and the generation of secondary inflammatory changes characteristic of CD.

The second hypothesis claims that Nod2 functions as a negative regulator of TLR2-mediated signalling and loss-of-function Nod2 mutations result in dysregulated TLR2-mediated Th1 inflammation in intestinal macrophages and/or DCs [67].

Finally, a third hypothesis coming from a study performed with knockin mice expressing the Nod2 frameshift mutant (L1007fsinsC) suggests this common mutation acts as a gain-of-function mutation [91]. In this study, macrophages from these knockin mice exhibit increased NF-κB activation and IL-1β secretion in response to MDP. However, these results are conflicting with those showing that monocytes from CD patients homozygous for this frameshift mutation exhibit a loss-of-function phenotype [14,88].

7. Conclusions and perspectives

For almost 10 years, Nod2 has been known as an innate cytosolic receptor able to trigger a RIP-2- and NF-κB-dependent pro-inflammatory and antibacterial response after sensing PGN from Gram-positive and -negative bacteria. Mutations in the gene encoding Nod2 in humans have been associated with Crohn's disease (CD). Mechanisms by which Nod2 variants can lead to CD development are still under investigation. The most admitted hypothesis suggests that the impaired function of Nod2 variants in intestinal epithelial and phagocytic cells results in deficiencies of epithelial-barrier function which subsequently lead to increased bacterial invasion and inflammation at intestinal sites.

Recent data have shown that Nod2 wild-type, unlike Nod2 variants, can induce autophagy in response to bacterial infection, thereby allowing efficient bacterial trafficking and MHC class II antigen presentation. These data reinforce the model of impaired epithelial-barrier since DCs expressing one variant could lead to inadequate generation of $CD4^+$ T immune responses, facilitating bacterial persistence and the generation of secondary inflammatory changes characteristic of CD. Further investigation will be required to elucidate the molecular mechanism involved in Nod2-mediated autophagy. These new results demonstrating a role of Nod2 in adaptative immune response have also allowed to better understand the MDP adjuvanticity known for more than 30 years.

Very recent results have expanded the function of Nod2 beyond detection of peptidoglycan. Indeed, Nod2 has been shown to confer responsiveness to viral ssRNA in terms of IRF3 activation and IFNβ production and, by this way, to contribute to antiviral response. Nod2-mediated IRF3 activation probably proceeds via a mechanism similar to RLRs (RIG-I and MDA-5) involving the mitochondrial adaptor protein MAVS. Like for MDP, further studies will be required to determine whether the mechanism of ssRNA sensing is direct or indirect, involving "bridging proteins".

Interestingly, Nod2 has been also shown to be exquisitely tuned to detect mycobacterial infections and mount a protective immunity against these pathogens. As CD is epidemiologically associated with mycobacterial infections [92], further studies are needed to determine whether mycobacteria can exploit variability in Nod2-mediated resistance to initiate or contribute to chronic inflammatory bowel disease.

Another recent study has shown that Nod2 signalling can also enhance the cross-priming of Ag-specific $CD8^+$ T cells by $CD8\alpha^+$ DCs, at least in part, by upregulating Ag cross-presentation pathway and costimulation via surface molecules. The mechanism by which Nod2 stimulation leads to increased expression of cross-presentation or costimulatory molecules, the involvement of RIP2, NF-κB and MAPKs and the behaviour of CD mutants need to be further explored. Nevertheless, these preliminary results might open onto the development of novel preventive and therapeutic applications for viral infections as well as cancer.

Finally, a T cell-intrinsic role of Nod2 independent on MDP recognition has been suggested. Nod2 would be involved in c-Rel-mediated IL-2 transcription in response to CD28 costimulation. While these data have to be further investigated, they elicit the question concerning the role of Nod2 in other cell types than monocytes/macrophages, dendritic and epithelial cells. Indeed, Nod2 has been shown to be expressed in neutrophils [93], preadipocytes [94], osteoblasts [95], renal tubular epithelial cells [96],...

In addition, Nod2 L1007fsinsC has been also associated with a growing number of malignant diseases, including early onset breast cancer [97], non-Hodgkin's lymphoma [98] and lung cancer [99]. The molecular mechanisms underlying these associations are still unclear.

Altogether, these new exciting discoveries raise many questions concerning the various potential roles of Nod2 and highlight its therapeutic potential.

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