Articles in PresS. Am J Physiol Gastrointest Liver Physiol (April 27, 2017). doi:10.1152/ajpgi.00330.2016

From sensing to shaping microbiota: insights into the role of NOD2 in intestinal homeostasis and progression of Crohn's Disease

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Key Words: IBD, Crohn's Disease, NOD2, MDP, Microbiota, Microbial Sensor.

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Financial Disclosure: The authors have no conflict of interest to declare.

ABSTRACT

NOD2 was the first susceptibility gene identified for Crohn's disease (CD), one of the major forms of inflammatory bowel disease (IBD). The field of NOD2 research has opened up many questions critical to understanding the complexities of microbiota-host interactions. In addition to sensing its specific bacterial components as a cytosolic pattern recognition receptor, NOD2 also appears to shape the colonization of intestinal microbiota. Activated NOD2 triggers downstream signaling cascades exampled by the NF-κB pathway to induce anti-microbial activities, however defective or loss of NOD2 functions incur a similarly activated inflammatory response. Additional studies have identified the involvement of NOD2 in protection against non-microbiota related intestinal damages as well as extra-intestinal infections. We survey on recent molecular and genetic studies of NOD2-mediated bacterial sensing and immunological modulation, and integrate evidences to suggest a highly reciprocal but still poorly understood crosstalk between enteric microbiota and host cells.

INTRODUCTION

The gastrointestinal (GI) tract contains a large population of commensal bacteria in symbiotic association with the human body. These bacteria aid in nutrient breakdown and are important for energy assimilation for the host. In healthy individuals, the intestinal epithelial cells (IECs) and the underlying immune cells tolerate commensal microbiota but respond to incursion by pathogenic microorganisms. Important mechanisms exist to maintain a delicate interaction between the host and microbiota. In addition to the innate barrier function presented by the mucosal layer, Pattern Recognition Receptors (PRRs), in particular members of the membrane bound Toll-Like Receptors (TLRs) [(58), reviewed in (48, 57)] and cytosolic nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs) play important roles in microbial recognition and immune modulation [reviewed in (9)].

Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is a member of the NLR family, consisting of two N-terminal caspase recruitment domains (CARDs), a central nucleotide binding domain (NBD), and several C-terminal leucine rich repeats (LRRs) (36). *NOD2* was the first susceptibility gene identified for Crohn's disease (CD) (20, 35), one of the major forms of inflammatory bowel disease (IBD). Due to its strong association with CD pathogenesis, NOD2-mediated bacterial sensing and immunological modulation have been extensively investigated over the past decade. By integrating insights from cell biology, immunology, and mouse genetics studies, we discuss the consensus view on how NOD2 may modulate the host-microbe crosstalk with emphasis on the mechanism of NOD2 bacterial sensing, activation of downstream signaling effectors, and impact of NOD2 on intestinal homeostasis and pathogenesis.

Bacterial Sensing by NOD2

The bacterial peptidoglycan (PGN), common to both gram-positive and gram-negative bacteria, is made up of β (1→4) linked N-acetyl glucosamine (GlcNAc) and N-acetyl muramic acid (MurNAc), which are further cross-linked by short peptides to establish the major constituent of the bacterial cell wall (43). Cleavage of PGN by specific glycoside hydrolases, such as lysozyme, releases muramyl dipeptide (MDP) that consists of a MurNAc linked to D-Ala and D-Glu (14, 21). NOD2 specifically recognizes and senses MDP (14, 15, 21). This MDP sensing capability by NOD2 has been suspected to be critical for modulating intestinal epithelia and microbiota interaction. In contrast to wild type NOD2, CD-associated *NOD2* variants show aberrant MDP sensing (14, 21), which could impair bacterial killing and cause microbial dysbiosis. Defective MDP sensing by NOD2 can ultimately compromise intestinal epithelial barrier and induce abnormal host immune response, resulting in exacerbated intestinal injury and CD pathogenesis, aspects which will be discussed through the course of this review.

The intracellular traffic and molecular delivery of MDP have been carefully investigated to understand NOD2-mediated MDP sensing and initiation of downstream NOD2 signaling. Surface plasmon resonance assay was used to demonstrate that MDP directly binds to NOD2 (15). The optimal NOD2-MDP binding took place within a pH range of 5.0-6.5 (15), a similar pH condition where dynamin-dependent MDP endocytosis occurs (26). Pull-down analyses carried out using biotinylated MDP and purified recombinant NOD2 (30) suggested that the NBD of NOD2, from amino acid 216 to 821, mediated the NOD2-MDP interaction (30). Whether NOD2-MDP binding is

93 influenced by NOD2's association with ATP/ADP has been controversial (15, 30).

94 The di/tripeptide transporter, PepT1, is predominantly found at the brush border
95 along the entire rat small intestine (34). Human peptide transporter, hPepT1, which is
96 mostly absent in normal colonic epithelia, showed an increased expression level in
97 inflamed colonic mucosa of CD patients (29). This aberrantly expressed hPepT1
98 exhibited uptake of MDP in human colonic Caco2 cells suggesting that MDP
99 internalization may be enhanced during colonic inflammation (52). Likewise, in vivo
100 study in MDP perfused rats suggested that MDP transport through intestinal PepT1
101 induces Nod2 mediated intestinal inflammation (28). In addition to PepT1, two other
102 endosomal oligopeptide transporters, SLC15A3 and SLC15A4, were shown to regulate
103 MDP transport via bacterial ligands internalized endosomes (31). SLC15A4 levels was
104 significantly elevated in inflamed colons of IBD patients (26), similar to hPepT1. The
105 human embryonic kidney (HEK) 293 cells stably expressing an IBD-associated NOD2
106 3020insC, which was truncated in the LRR domain, showed greatly reduced NOD2-
107 SLC15-phagosome interactions, accompanied by an attenuated pathogen sensing (31).
108 Thus, the LRR domain of NOD2 was likely responsible for its interaction with above
109 SLC15 transporters and pathogen sensing.

110 Internalization of PGN, of which MDP is a structural unit, could also be accomplished
111 by direct shedding of PGN from invasive species such as *Shigella*. This was shown to
112 trigger NF-κB pathway activation through NOD1 rather than NOD2 and TLR in HEK293
113 cells (32). Similar activation of NF-κB pathway by NOD1 in gastric epithelial cells could
114 also be induced by PGN that was delivered through the type IV secretion system from
115 the invasive Gram-negative *Helicobacter pylori* (2). In addition, outer membrane

vesicles (OMVs) from Gram-negative bacteria, such as *Vibrio cholerae*, induced NOD1/2 mediated responses in HEK 293T cells (7). Recent *in vivo* studies demonstrated that Polysaccharide A in OMVs from *Bacteroides fragilis* required mouse Nod2 and Autophagy-related 16 like 1 (Atg16l1) to stimulate immune response and suppress inflammation in 2,4-dinitrobenzene sulfonic acid (DNBS)-induced colitis model (8). Moreover, PGN in the intestinal lumen was shown to be captured by calcium phosphate nanominerals and transported via M cells to antigen-presenting cells in the Peyer's patch in both human and murine tissues (38). Peyer's patches are aggregated lymphoid follicles present in the gut-associated lymphoid tissue and surrounded by follicle-associated epithelium which contains the M cells (23). In summary, the existence of multiple cellular types as well as internalization pathways appear to be critical for commensal bacterial sensing and pathogen surveillance during homeostasis and intestinal inflammation.

In addition to providing a direct signal to host cells via NOD2 mediated sensing, MDP also influences the anti-bacterial functions of host intestinal cells. Paneth cells store antimicrobial peptides (AMP) such as lysozyme in dense core vesicles (DCVs) before secreting them into intestinal lumen. MDPs induce the localization of Nod2 to these DCVs in mouse Paneth cells, followed by a recruitment of leucine rich repeat kinase 2 (Lrrk2) and Rab2a. This process was critical for sorting lysozyme into DCVs in Paneth cells and for protecting the intestinal epithelium (60). Of note, *LRRK2* is also a susceptibility gene for CD (6) suggesting that defective sorting of AMPs by MDP-NOD2 may be, in part, responsible for CD pathogenesis.

MDP was previously considered to be the minimal bioactive PGN motif; however

recent studies showed that GlcNAc itself triggered inflammasome activation through hexokinase dissociation in the cytoplasm (56). This finding will stimulate further research into the PGN moieties of intestinal microbiota and the mechanisms contributing to the maintenance and disruption of mucosal and microbial homeostasis.

NOD2's Downstream Signaling Events

The cellular effectors and signaling pathways downstream of NOD2 have been studied to understand its contribution to the maintenance of mucosal immunity. Receptor interacting serine/threonine protein kinase 2 (RIPK2) is one of the well-studied interaction partners of NOD2 and NOD2-RIPK2 downstream signaling cascade can be briefly summarized as follows: MDP stimulation initiates NOD2 binding to RIPK2 (10), RIPK2 then gets ubiquitinated and recruits TAK1 and IKK complex (16), followed by phosphorylation of IκBα, to activate NF-κB pathway (16, 36).

Studies delineating the delivery routes of MDP also examined the NOD2 signaling mechanism initiated by MDP detection. Upon sensing of MDP from internalized invasive *Salmonella typhimurium* (S. Tm), NOD2 in HEK293 cells recruited RIPK2 to endo-lysosomes to establish the signaling complex required for responding to pathogens (31). In an exacerbated intestinal injury model using MDP-perfused rats, RIPK2 was implicated as the NOD2 downstream signaling factor (28).

Identification of the positive and negative regulators of NOD2 pathway is imperative to understanding NOD2 cellular function. Interferon regulatory factor 4 (IRF4) is a negative regulator of NOD2 induced NF- κB signaling (55). In human dendritic cells, IRF-4 expression stimulated by NOD2-mediated MDP sensing inhibits polyubiquitination of RIPK2 which subsequently downregulates NF-κB expression. This results in reduced

colonic inflammation in experimental colitis models (55). Being one of NOD2's downstream effectors, NF-κB in physiological conditions induces transcription of inflammatory genes upon its nuclear translocation. The IRF-4 mediated check on NF-κB expression contributes to the importance of regulation of intestinal immune responses in pathological conditions.

A genome wide siRNA study in HEK293 cells stably expressing NOD2 and NF-κB luciferase reporter identified a plethora of NOD2 effectors and regulators of NOD2-NF-κB signaling related to autophagy, ubiquitination, and endosomal sorting pathways (54). Fifteen of the identified genes including IL-21, IL-19 and STAT3, were associated with CD risk, supporting the involvement of NOD2 and its regulators in CD pathogenesis (54). Likewise, RNAi screens were done to identify additional positive and negative regulators of NOD2 pathway (27). The FERM and PDZ domain-containing 2 (FRMPD2) was shown to be critical for NOD2 localization at plasma membrane and found to positively regulate NOD2 signaling from the basolateral domain of IECs. FRMPD2, whose expressional level reduced significantly in CD patients, was placed upstream of RIPK2 (27).

A recent addition to the above NOD2 downstream signaling network is the tumor progression locus 2 (TPL2), a homolog of the human MAP3K8. An IBD-risk locus (rs1042058) is present in the *TPL2* region (17). In monocyte-derived macrophages (MDM), IKK complex is required to induce NOD2-mediated phosphorylation of TPL2, which subsequently activates ERK and NF-κB, as well as the secretion of IL-1β and IL-18 cytokines (17). In both healthy controls and CD patients, rs1042058 GG carrier MDMs exhibited upregulating of TPL2 and downstream cytokine secretion when

compared to AA carriers (17). So, the authors report that rs1042058 GG IBD-risk polymorphism in *TPL2* may act as a gain-of-function leading to an increase in TPL2 levels and enhanced NOD2-mediated cytokine secretion. These studies on regulators of NOD2 signaling cascade contributed towards an understanding of NOD2-mediated maintenance of intestinal homeostasis.

Another important NOD2 interaction partner is CARD9, also a CD susceptibility gene (62). Upon stimulation, NOD2 binds and utilizes CARD9 to regulate innate immune responses (18). *Card9*$^{-/-}$ mice infected with *Listeria monocytogenes* exhibited an increased bacterial load with a delayed induction of strong proinflammatory responses (18). Thus, several NOD2 downstream signaling molecules and pathways have been uncovered with complicated positive and negative feedbacks. If future functional studies can assess their individual contributions to epithelial homeostasis and IBD progression, and carefully delineate primary and secondary effectors, relevant targets may be selected for disease intervention.

Impact of NOD2 on Intestinal Epithelia and Immune Cells

The crypt based columnar (CBC) intestinal stem cells (ISCs) give rise to the entire differentiated population of IECs. RT-PCR analysis carried out on sorted cells from crypts of Lgr5-EGFP knockin mice revealed that CBC cells had a 5-fold higher level of Nod2 mRNA than the neighboring Paneth cells **(Figure 1)** (33). To further decipher the role of NOD2 expression in CBC cells, organoids were used as a model of study. ISCs from isolated murine crypts can be grown in matrigel supplemented with essential growth factors to form self-sustained organoids that resemble many features

of intestine (42). ISCs sorted and isolated from wild type mice generated more organoids than ISCs from *Nod2*-deficient mice (33). While the addition of MDP provided a further increase in organoids yielded from wild type stem cells, no difference was observed in the mutant condition. Moreover, crypts from doxorubicin treated wild type mice yielded more organoids upon MDP stimulation whereas organoids grown from *Nod2*-deficient mice injected with doxorubicin failed to recover even on addition of MDP. Doxorubicin is a DNA intercalating agent that is detrimental to ISCs. These results suggest that Nod2 mediated MDP sensing is critical to stem cell survival and renewal (33). In a similar study performed on colonic epithelial cells (CECs), Nod2 mRNA levels was augmented in proliferating crypt epithelial cells compared to the differentiating cells (12). From an *in vivo* context, wild type mouse CECs showed increased proliferation and provided protection from invasion by *Salmonella enterica serovar typhimurium,* compared to CECs of *Nod2*-deficient mice. Addition of MDP could not rescue the cell number of *Nod2*-deficient CECs in comparison to the increase observed in MDP treated wild type CECs in vitro. Thus, Nod2 can promote colonic epithelial cell growth in response to MDP sensing.

Nod2 global deletion has no effect on Paneth cell numbers (45, 50). This was corroborated by another study where Nod2 deficiency affected the expression levels of AMPs and mucin independent of Paneth and goblet cell number in the ileum (1). Specifically, Nod2 deficiency reduced Paneth cell specific Reg3β and Reg3γ, but not cryptdin, while it increased goblet cell markers, Muc4, intestinal trefoil factor, and Fc-γ binding protein (1). However, during Paneth cell differentiation of Caco2 cells, NOD2 signaling through MDP decreased the expression of other Paneth cell specific AMPs

including the enteric α-defensin (HD)5, HD6, lysozyme, and secretory phospholipase A2 (sPLA2) (49). In contrast, several studies reported that Nod2 deletion does not impact secretion of any AMP (41, 45) or the bactericidal activity of α-defensins (45). A study using resection specimens from CD patients showed that the degree of abnormality in Paneth cell morphology was proportional to the number of *NOD2* risk variants (51). But, Ramanan *et al*. (40) observed that Paneth cell morphology did not alter in *Nod2*-deficient mice. In addition, NOD2 sensing of indigenous bacteria was shown to affect lysozyme sorting into the DCVs in Paneth cells (60) **(Figure 1)**. This sensing was proposed to facilitate localization of Nod2, Lrrk2, and Rab2a to the DCVs, thereby preventing degradation of lysozyme in lysosomes (60).

NOD2 has also been implicated in regulating goblet cell morphology and mucin secretion **(Figure 1)**. Mucin forms a major component of the mucosal layer that establishes a barrier between host epithelium and microbiota and provides the first line of defense in case this balance is compromised. *Nod2*-deficient mice were characterized by reduced Muc2 expression and decreased number of mucin granules per goblet cell (40). Wang *et al.* (53) challenged wild type and *Nod1*/*Nod2* double-knockout mice with commensal parasite *Trichuris muris*, and demonstrated that sensing of the microbiota by Nod1 and Nod2 was important for increasing goblet cell number and mucin secretion, which might help to counter the parasitic load in the intestine.

NOD2 is expressed by at least two distinct cell populations in the intestine: IECs and hematopoietic immune cells (3, 22, 25). By using reciprocal bone marrow transfer assays between *Nod2* wild type and deficient mice, Alnabhani *et al*. (3) demarcated that IEC specific Nod2 impacted microbial dysbiosis and antimicrobial secretion, whereas

hematopoietic cell specific Nod2 influenced the immune response and barrier function. They attributed microbial imbalance to IEC Nod2 whereas another study implicated hematopoietic Nod2 for microbial imbalance (40), a discrepancy that requires further investigation. Thus, whether NOD2 in different cell types play distinct roles for intestinal-microbial homeostasis is still not clear but warrants further investigations.

CD has been characterized by an increase in T cell population and pro-inflammatory responses (44). *Nod2*-deficient mice with acute T-cell activation using T cell activating anti-CD3 monoclonal antibody presented disturbed intestinal crypt-villous architecture and increased levels of IL-17A, TNF-α, and IFN-γ, compared to wild type mice (59). On treatment with broad-spectrum antibiotics, T- cell activated *Nod2*-deficient mice could restore normal crypt architecture and cytokine levels 3 days after anti-CD3 injection. Further investigation revealed that acute T-cell activation in cell lineage specific deletion of Nod2 by either *Villin*-Cre or by *Lyz2*-Cre did not affect the intestinal architecture. However, the *Lyz2*-Cre mice model exhibited an increase in cytokines such as TNF-α and IL-22, similar to the *Nod2*-deficient mice. Collectively, this suggests that Nod2 sensing of gut microbiota modulates intestinal homeostasis following acute T-cell activation (59). Previously, Barreau *et al.* (5) demonstrated that antibodies against $CD4^+$ T cells and IFN-γ levels in the Peyer's patch of *Nod2*-deficient mice could reduce the elevated transcellular permeability and bacterial translocation across Peyer's patch by regulating myosin light chain kinase (MLCK) expression. Moreover, inhibition of MLCK in these mice attenuated levels of $CD4^+$ T cells and IFN-γ along with rescuing the barrier defect, thereby suggesting a reciprocal interaction between immune and epithelial cells regulated by Nod2. Collectively, NOD2 seems to modulate the intestinal

epithelial differentiation and the mucosal defense mechanisms **(Figure 1)**.

Impact of NOD2 on Microbiota

Nod2-deficient mice showed a significant expansion of *Bacteroides vulgatus,* an anaerobic commensal bacterium compared to the wild type mice (40). This expansion was associated with increased IFN-γ expressing $CD8^+/TCR\gamma\delta^+$ intraepithelial lymphocytes (IELs), despite a decrease in the total number of IELs and defective goblet cell morphology. Treatment of the microbiota by metronidazole, an antibiotic against anaerobic bacteria, restored the epithelial morphology and cytokine expression (40). The phenotype of decrease in IEL population in *Nod2*-deficient mice has been also been reported in a previous study (22). The paper on expansion of *B. vulgatus* supports the notion that NOD2 mediates crosstalk between intestinal microbiota and immune system. Ramanan *et al.* (39) further investigated that CD pathophysiology characterized by expansion of *Bacteroides* species due to *Nod2* mutation could be treated with a helminth infection. Colonization with helminthes ameliorated all abnormalities with a strong Th2 response and expansion of *Clostridiales* over *Bacteroides* (39). Taken together, NOD2 mutation leads to exacerbated intestinal inflammation associated with microbial dysbiosis and specific expansion of the *Bacteroides*.

Pseudomonas fluorescens (*P. fluorescens*) antigen was detected in the serum of almost 54% CD patients (47). *P. fluorescens* infection enhanced IL-1β production and MLCK levels, thereby increasing the paracellular permeability in ileal mucosa (4). Nod2 secreted by macrophages is required for inducing IL-1β production in response to *P. fluorescens*; however, addition of MDP was able to attenuate the defect in epithelial barrier and IL-1β production (4).

The response of NLR sensing of intestinal microbiota is not restricted to local homeostasis. Recently, long range effects of NLR signaling on host defense against pathogenic microorganisms have been reported. Specifically, NLR stimulation in the intestine stimulates bacterial killing in the lungs through reactive oxygen species released by alveolar macrophages. In the absence of NLR stimulation, antibiotic treated mice weakly stimulate immune response in the lung tissue as observed through IL-6 and TNF-α levels (11).

The protective function of NOD2 extends to even non-microbiota related conditions, such as ischemia/reperfusion mediated intestinal damage. In response to this type of damage, Nod2 dependent sensing of commensal bacteria restores epithelial morphology in IECs through induction of autophagy (37). Nod2 signaling is also being considered for intestinal sepsis treatment. Septic rat intestines treated with Saikosaponin A showed reduced Nod2 expression accompanied by an inhibition of pro-inflammatory cytokines IL-6 and TNF-α via NF-κB pathway (61). It is interesting to note that NOD2 signaling in the intestinal surface is being studied for its protective function in conditions apart from CD. NOD2 is vital for defense at the intestinal barrier as well as at distant sites. Future studies should identify mechanisms by which NOD2 establishes such an extensive regulation.

Collectively, studies of *Nod2*-deficient mice have documented a reduced α-defensin expression, a changed microbiota composition (24), as well as a significant expansion of *Bacteroides vulgatus* in these knockout animals (40). However, some studies have shown that by controlling for the genetic background of the mice, in this case C57BL/6, the housing conditions and adequate number of mice, the above

differences in defensin expression and microbiota composition were negated (41, 45, 46). To resolve the impact of Nod2 on intestinal microbiota composition, Al Nabhani *et al*. (1) used embryo transfer approach to minimize variability of environmental factors. Interestingly, *Nod2* wild type and *Nod2*-deficient embryos transferred to a *Nod2* wild type mother shared similar gut microbial composition to Nod2 knockout mice rather than their wild type mother. Thus, it proved that Nod2 deletion is linked with dominant and transmissible microbial dysbiosis. This microbial dysbiosis further affects the secretion of AMPs and mucin but does not impact immune response and gut epithelial barrier function. Similarly, mice lacking another NLR family member, *Nlrp6* harbored an impaired microbiota (13). Wild type mice cohoused with *Nlrp6*$^{-/-}$ mice exhibited dysbiotic microbiota and increased susceptibility to DSS- induced colitis and AOM-DSS induced colorectal cancer similar to single-housed *Nlrp6*$^{-/-}$ mice rather than single-housed wild type mice (13, 19). These results shed light on the importance of experimental design to dissect role of host gene expression on gut microbiota, intestinal epithelial and host immunological responses.

CLOSING REMARKS

NOD2 sensing of the intestinal microbiota allows nuclear translocation of NF-κB that initiates transcription of inflammatory cytokines. While *NOD2* mutations disrupt the balance between gut microbiota, immune response, and intestinal epithelium, which also results in exacerbated inflammation and eventually IBD. Ramanan *et al.* (40) showed that small intestinal abnormalities and inflammation in *Nod2*$^{-/-}$ mice were dependent on expansion of *B. vulgatus,* providing a possible explanation to the complex relationship between NOD2 and immune response to intestinal microbiota. Future

studies will acquire insights into such mechanisms and their role in disease IBD pathophysiology. It is also interesting to define the potential contribution of NOD2-mediated cellular autophagy to disease progression. The observation that intestinal NOD2 signaling may transcend intestinal barrier to distant sites indicate that monitoring intestinal bacteria by this pathway may be crucial for systemic microbial surveillance for human health. This makes it a plausible target for improving the overall well-being in clinic.

Acknowledgement:

This work is supported by National Institutes of Health (NIH) grants (DK102934, DK085194, DK093809, CA178599], an Initiative for Multidisciplinary Research Teams (IMRT) award from Rutgers University, National Science Foundation (NSF)/BIO/IDBR [1353890], and a Research Scholar Grant [RSG-15-060-01-TBE] from the American Cancer Society to N.G.

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Figure Legend

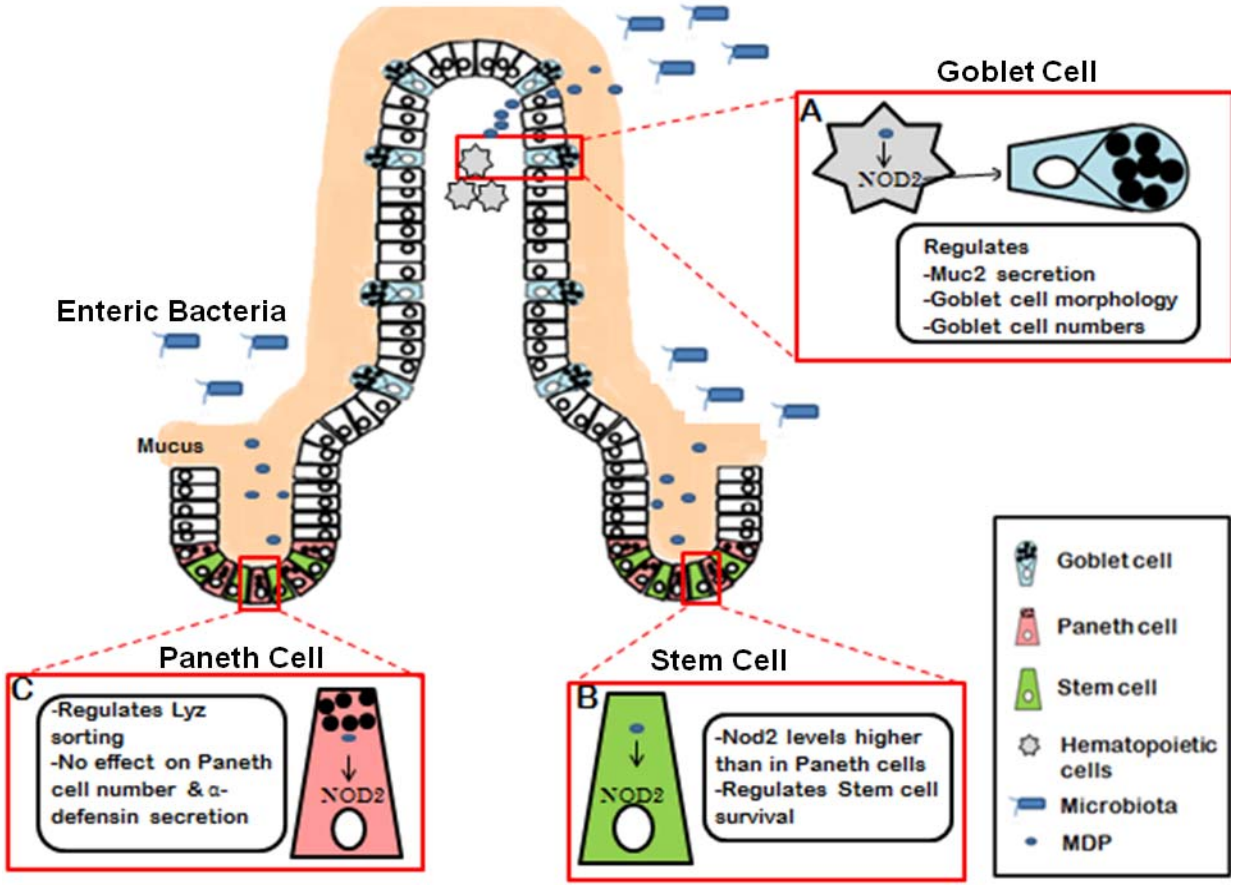
Figure 1: NOD2-mediated bacterial sensing and modulation of intestinal epithelial homeostasis.

Binding of bacterial MDP to cytosolic NOD2 receptors appears to have comprehensive influences on intestinal mucosal homeostasis.

(A) Incursion of pathogenic microorganisms or detrimental expansion of commensal bacteria may be sensed by hematopoietic NOD2-expressing cells that regulate goblet cell number, morphology, as well as the expression and secretion of anti-microbial gene products such as mucin 2.

(B) Crypt based columnar (CBC) stem cells express higher levels of NOD2 compared to the Paneth cells. MDP sensing by NOD2 appears to influence stem cell renewal or/and survival.

(C) NOD2, as well as NOD2-mediated bacterial sensing, may regulate lysozyme sorting in Paneth cells, although the expression of several anti-microbial peptides, such as α-defensin, is not dependent on NOD2.



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612 **Figure 1. NOD2-mediated bacterial sensing and modulation of intestinal epithelial**

613 **homeostasis.**

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