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Review

Role of Nlrp6 and Nlrp12 in the maintenance of intestinal homeostasis

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There has been significant interest in understanding how interactions between the host immune system and the gut microbiota regulate intestinal homeostasis. Recent data suggest that the Nod-like receptor (NLR) family of PRRs regulate both the composition of the gut microbiota and innate immune signaling pathways that prevent pathologic intestinal inflammation and tumorigenesis. In this review, we will focus on NLRP6 and NLRP12, two members of the NLR family that have emerged as important players in the maintenance of intestinal homeostasis, and discuss the signaling pathways engaged by these receptors as well as the current models of how these receptors protect against the development of colitis and tumorigenesis.

Keywords: Cancer · Immune regulation · Immune responses · Inflammation · Innate immunity

Introduction

Since the discovery that host bacterial recognition pathways are critical for maintaining intestinal homeostasis [1], there have been numerous studies demonstrating how members of the Nod-like receptor (NLR) family play an important role in both promoting host defense against invasive pathogens and reducing host susceptibility to chemically-induced colitis and subsequent tumorigenesis [2–9]. NLRs have been traditionally considered as PRRs, in that they are activated in response to conserved structural motifs found in microbes or PAMPs, such as peptidoglycan or flagellin [10, 11]. More recently, NLRs, particularly NLRP3, have been implicated in recognizing endogenous stimuli related to cellular injury, or damage-associated molecular patterns, which can result in sterile inflammation [12]. NLRs are characterized by a tripartite structure consisting of (i) a variable N-terminal protein–protein interaction domain, (ii) a central nucleotide-binding oligomerization (NOD) domain that mediates the self-oligomerization occurring during activation [13], and (iii) a C-terminal leucine-rich repeat (LRR) involved in ligand specificity. The N-terminal domain of an NLR can be defined as a caspase recruitment domain (CARD), pyrin domain (PYD), acidic, transactivating domain or baculovirus inhibitor repeat. There are at least 23 identified human NLRs, and 34 NLRs have been identified in mice. A standardized nomenclature system [14] categorizes the NLR family into four subfamilies based on the type of N-terminal domain. Two NLRs in particular (NLRP6 and NLRP12), both highly expressed in the intestine, act as negative regulators of intestinal inflammation and tumorigenesis [7–9, 15, 16]. NLRP6 and NLRP12 belong to the subfamily of NLRs that contain an N-terminal (PYD), which can interact with other PYD-containing proteins that are important for downstream signaling events. Multiple members of the NLR family, including NOD1, NOD2, NLRC4, and NLRP3, have been implicated in maintaining intestinal homeostasis [2, 4, 15, 17, 18]. These members are relatively well-characterized and the nature of their upstream ligands have been identified. In this review, we will focus on the recently recognized roles of NLRP6 and NLRP12, two NLRs whose upstream agonist has not yet been identified, in the protection against intestinal inflammation and tumorigenesis.

NLRP6 and NLRP12 participate in multiple signaling pathways

Early in vitro studies have implicated both NLRP6 and NLRP2 in inflammasome formation [19, 20]. Inflammasomes are

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multiprotein complexes whose assembly is mediated by the adaptor protein apoptosis-associated speck-like protein (ASC). ASC possesses both a carboxy terminal, CARD, and a PYD, and, therefore, is capable of interacting with NLRs that also contain a PYD domain through homophilic protein–protein interactions. The current model of inflammasome assembly hypothesizes that NLR activation by its agonist results in oligomerization through the NOD domain of the receptor. Subsequently, through CARD–CARD and PYD–PYD protein–protein interactions, a large macromolecular complex is assembled, which serves as a platform for procaspase-1 recruitment. After recruitment to the inflammasome complex, procaspase-1 self-cleaves into active caspase-1 [21]. Caspase-1 then cleaves pro-IL-1β and pro-IL-18 into their mature and active forms [22].

Both NLRP6 and NLRP12 have been demonstrated to colocalize with ASC in a characteristic speckled pattern within the cytoplasm. Colocalization is dependent on the presence of the PYD in both NLRP6 and NLRP12 [19,20]. Thus, both NLRP6 and NLRP12 have been considered as members of the inflammasome. However, in these studies, which employed overexpression of NLR proteins, a direct physical interaction between ASC and either NLRP6 or NLRP12 could not be demonstrated. This may reflect the poor solubility of ASC upon oligomerization after activation [23]. Alternatively, the interaction between ASC and NLRP6 or NLRP12 may be highly transient in nature. Nonetheless, coexpression of ASC and NLRP6 in COS-7L cells in vitro resulted in increased IL-1β production that was dependent on the availability of caspase-1 and the NLRP6 PYD [20]. Similarly, NLRP12 has been demonstrated to cooperate with ASC to promote caspase-1 activation and production of IL-1β in a PYD-dependent manner [19]. Consistent with a role for NLRP12 in the production of IL-1β and IL-18, NLRP12-deficient mice have increased mortality to infection by an attenuated strain of *Yersinia pestis* in comparison to WT mice [24]. In this model, IL-1β production by bone marrow-derived macrophages (BMDMs) and serum IL-18 levels are decreased in NLRP12-deficient mice. However, IL-1β and IL-18 production are not completely abolished, suggesting redundancy in cytokine production pathways. Reduced IL-18 production was also observed in NLRP6-deficient mice, which was associated with increased susceptibility to chemically-induced colitis and colitis-associated tumorigenesis (discussed further below) [7–9]. Evidence for the involvement of NLRP6 and NLRP12 in inflammasome formation and activation remains largely derived from in vitro studies in which these proteins are overexpressed. To date, the ligand that stimulates NLRP6 or NLRP12 activity remains unknown. Thus, direct evidence that NLRP6 or NLRP12 activate the inflammasome under physiologic conditions in vivo has been difficult to obtain.

In addition to a potential role in inflammasome signaling, in vitro studies have also suggested that NLRP6 and NLRP12 are positioned upstream of NF-κB and MAPK in cell signaling pathways (Fig. 1). Although NLRP6 and NLRP12 are both unable to activate NF-κB alone in luciferase reporter assays, there is synergistic activation of NF-κB together with ASC when either NLRP6 or NLRP12 were co-transfected with ASC in 293T cells [19,20]. More recently, NLRP6 was shown to be a negative regulator of canonical NF-κB signaling. Specifically, stimulation of NLRP6-deficient BMDMs with bacterial TLR-2 and TLR-4 ligands, results in significantly upregulated NF-κB and MAPK (ERK) signaling, culminating in increased cytokine production such as TNF-α and IL-6 [25]. Consequently, NLRP6-deficient mice are resistant to systemic bacterial infection, resulting from greater induction of proinflammatory cytokines, increased bacterial clearance, and survival [25]. However, whether NLRP6 also participates in host defense against gastrointestinal infections remains to be investigated.

NLRP12 has also been shown to downregulate NF-κB responses to TLR agonists, and its expression is reduced in response to TLR stimulation in vitro in THP-1 cells [16,26]. However, it is unclear whether NRLP12 acts primarily on the noncanonical versus canonical pathway. Early in vitro studies implementing siRNA knockdown of NLRP12 expression or protein overexpression suggested that NLRP12 negatively regulates the noncanonical NF-κB pathway. More specifically, NLRP12 directly associates with the NF-κB-inducing kinase (NIK) through its NOD and LRR domains, leading to proteasome-mediated degradation of NIK [27]. NIK induces NF-κB activation by inducing the phosphorylation of the noncanonical NF-κB effector p100, which is then processed into the p52 subunit that transcriptionally activates p52-regulated genes. Other in vitro protein overexpression studies show that NLRP12 also downregulates TLR-mediated pathways through association with IRAK-1 [26]. Furthermore, knockdown of NLRP12 expression by siRNAs in THP-1 cells results in higher expression of IL-6 in response to TLR ligands as well as to *Mycobacterium tuberculosis* [26]. However, these in vitro results were not reproduced in in vivo models of pulmonary infection with *M. tuberculosis* or *Klebsiella pneumonia* [28]. Specifically, although TNF-α and IL-6 production in bone marrow-derived DCs (BMDCs) were significantly elevated in NLRP12-deficient cells compared with that in WT cells, NLRP12-deficient mice did not exhibit any differences in disease outcome compared with WT mice [28], suggesting that other NLRs may be able to substitute for NLRP12. In the gastrointestinal tract, NLRP12 negatively regulates inflammatory responses during chemically induced colitis [15, 16] (discussed further below). Although these studies point to a role for NLRP12 in regulating NF-κB, at least one study has demonstrated no significant difference in NF-κB activation in response to *Yersinia* or TNF-α [24]. It is therefore possible that the nature of the stimulus and the specific context in which it is given is important in revealing a significant function for NLRP12 in NF-κB regulation.

NLRP6 maintains intestinal homeostasis by regulating the gut microbiome

A significant role for the gut microbiota in dictating host susceptibility to intestinal disease has emerged. This is highlighted by studies showing transmissibility of intestinal disease by co-housing of laboratory mice [7, 17, 29, 30]. Dextran sulfate sodium (DSS) can be used to induce a disease model of colitis, characterized by mucosal ulceration and disruption of the epithelial barrier that results in commensal-driven inflammation arising from

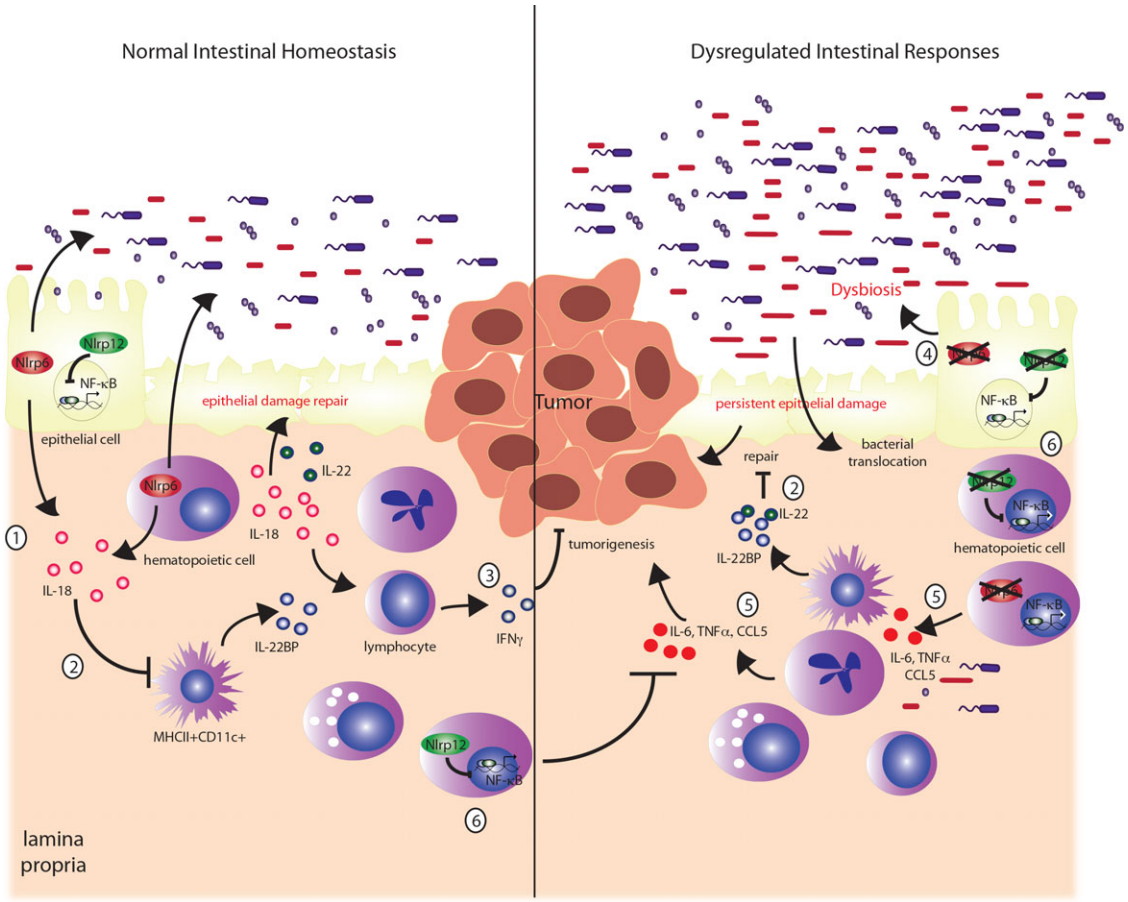

Figure 1. Multiple mechanisms involved in suppressing inflammation and tumorigenesis by NLRP6 and NLRP12. NLRP6 regulates IL-18 production, which helps to maintain a healthy microbiome and promotes epithelial repair (1). IL-18 also downregulates IL-22BP (2), thereby allowing IL-22 to participate in epithelial proliferation and repair of epithelial damage. NLRP6-deficiency is also associated with persistent IL-22BP production by primarily MCHII^{+}CD11c^{+} cells [35], which results in inhibition of the reparative activity of IL-22 (2). IL-18 potentially upregulates IFN-γ production, which promotes antitumor responses (3). In the absence of Nlrp6, the microbiome is altered, leading to the accumulation of potentially colitogenic bacteria, or dysbiosis (4), which can upregulate inflammatory factors, including CCL5 to promote inflammation and tumorigenesis especially in the presence of a damaged epithelial barrier (5). The persistence of epithelial damage allows bacterial translocation into the colonic mucosa to stimulate an aberrant inflammatory response by immune cells that promote inflammation and tumorigenesis (5). Similarly, NLRP12 negatively regulates NF-κB responses (6) although there are discordant observations regarding canonical versus noncanonical NF-kB pathways. NLRP12 deficiency is also associated with increased inflammation followed by tumorigenesis related to uncontrolled NF-κB signaling in either hematopoietic and/or nonhematopoietic cells (6).

bacterial translocation into the mucosa. Mice deficient in several of the NLRs have increased susceptibility to DSS-induced colitis associated with microbiome changes distinct from that in WT mice [7, 17, 31], including NLRP6. NLRP6-deficient mice developed more severe DSS-induced colitis compared with WT mice; however, after co-housing WT and NLRP6-deficient mice together for 4 weeks, WT mice acquire the same phenotype as NLRP6-deficient mice [7]. Microbiome profiles obtained by 16S rRNA sequencing revealed distinct microbial communities in WT and NLRP6-deficient mice [7]. In fact, NLRP6-deficient and ASC-deficient mice exhibit similar bacterial community profiles, suggesting an inflammasome-dependent regulation of the composition of the gut microbiota. Interestingly, mice deficient in other inflammasome-related NLRs, such as NLRC4, NLRP10, NLRP12, and AIM2, did not affect the susceptibility of WT mice to colitis after co-housing [7]. Notably, bacterial phylotypes that are significantly different between WT and NLRP6-deficient mice include *Prevotella,* and a member of the family *Prevotellaceae* as well as the phylum TM7 [7]. Antibiotic treatment of *Nlrp6*$^{-/-}$ mice results in the disappearance of both *Prevotellaceae* and TM7 and is associated with the amelioration of DSS-induced colitis [7]. IL-18-deficient mice, but not IL-1R-deficient mice, are also able to transmit increased susceptibility to colitis to WT mice. Moreover, the *Nlrp6*$^{-/-}$ mice used in this study have decreased levels of IL-18 in the serum and colon at baseline, suggesting that the altered microbiome in NLRP6-deficient mice is related to defective IL-18 production [7]. Changes in the microbiome not only affect susceptibility to colitis, but also to inflammation-associated tumorigenesis under the AOM/DSS

model, in which mice are given an intraperitoneal injection of the carcinogen azoxymethane (AOM) followed by multiple rounds of DSS treatment [7, 30]. This phenotype is shared by both NLRP6-deficient and ASC-deficient mice and is transmissible to WT mice. The *Nlrp6*$^{-/-}$ mice used in both colitis and tumor development studies have elevated levels of CCL5 at baseline, associated with abnormal crypt hyperplasia and increased immune cell infiltration. When CCL5-deficient mice are cohoused with *Nlrp6*$^{-/-}$ mice, CCL5-deficient mice do not develop increased colitis or tumors, suggesting that the dysbiotic microbiota promotes both inflammation and tumorigenesis that is mediated by CCL5 (Fig. 1) [7, 30].

The above results are exciting in that they suggest that certain bacteria within the microbiota are potentially colitogenic or tumorigenic and the accumulation of these bacteria is regulated by NLRP6 and the inflammasome. This indicates possible therapeutic targets for the treatment or prevention of inflammatory bowel disease and colitis-associated colorectal cancer. However, the results of these studies should also be interpreted with some caution. First, the community structure of the gut microbiome in IL-18-deficient mice remains distinct from that of NLRP6-deficient mice, and therefore it is likely that additional factors contribute to the microbiome of NLRP6-deficient mice. In addition, *Asc*$^{-/-}$ mice cross-fostered with WT mothers do not develop more severe colitis compared with noncross-fostered *Asc*$^{-/-}$ mice, suggesting that ASC deficiency alone is insufficient for the development of a dysbiotic microbiome capable of increasing disease severity, and that environmental rather than genetic factors contribute to microbiome composition. Finally, alterations in colon crypt morphology and impaired serum levels of IL-18 were not observed in colonies of NLRP6-deficient mice at other institutions [8], suggesting that there may be colony-dependent effects. As previous studies evaluating TLR-deficient mice determined that the composition of the microbiome is largely dependent on maternal transmission of the microbiota from isolated mouse colonies rather than on any TLR defect [32], it remains possible that the observed microbiome changes in NLRP6-deficient mice [7] are related more to differences in ancestry rather than to an immune defect. Additional experiments with germfree WT and N*lpr6*$^{-/-}$ mice may provide additional insight into the relationship between NLRP6 and the gut microbiome.

NLRP6 has also been implicated in the regulation of the gut microbiome to affect stress-induced small intestinal disease in the water-avoidance stress (WAS) model of irritable bowel syndrome [32], in which mice are regularly placed on a platform surrounded by water for 10 days. Inflammation, interestingly, is limited to the small intestine and is associated with a reduction in NLRP6 expression in the epithelium and decreased IL-1β and IL-18 production [33]. This phenotype is also transmissible to co-housed WT mice not subjected to WAS, and 16S rRNA sequencing revealed distinct microbiome changes in the luminal contents of the small intestine with changes in relative abundance in multiple bacterial families. Pretreatment of mice with a *Lactobacillus*-containing probiotic significantly reduced the severity of WAS-induced intestinal inflammation and also "normalized" the microbiome. It remains to be determined whether the observed microbiome changes are mediated by NLRP6 signaling and directly cause intestinal inflammation, rather than are a consequence of it.

NLRP6 maintains intestinal homeostasis by regulating epithelial repair and proliferation

As discussed above, impaired IL-18 production may be associated with the development of an altered microbiota, or dysbiosis, that predisposes to colitis and tumorigenesis. However, IL-18 also promotes epithelial repair by (i) MyD88 signaling [1, 34]; and (ii) downregulating the expression of IL-22 binding protein (IL-22BP) [35] (Fig. 1). IL-22BP interacts with IL-22, a cytokine which promotes epithelial proliferation during wound healing, to prevent it from binding to its cognate receptor [36, 37]. Indeed, IL-18-deficient mice have more severe DSS-induced colitis, which is associated with impaired epithelial repair [4]. Consistently, mice deficient in caspase-1 and ASC also have decreased IL-18 production and greater severity of colitis compared with WT mice, a condition which can be rescued by the administration of recombinant IL-18 to *caspase-1*$^{-/-}$ mice [4]. Caspase-1 and IL-18-deficent mice also develop more inflammation-associated tumors, with the former exhibiting decreased IFN-γ and STAT1 signaling, both of which are important for antitumor responses [5]. NLRP6-deficient mice also have decreased IL-18 levels in the serum and in the colon tissue during the acute inflammatory response to DSS in the AOM/DSS model, which may explain defects in epithelial restitution observed in these mice [8]. Consistent with this hypothesis, NLRP6-deficient mice also fail to downregulate IL-22BP after DSS-induced injury in the AOM/DSS tumor model [35]. Interestingly, although studies suggest that NLRP6 is expressed primarily in the epithelium [7], bone marrow chimeric experiments suggest that Nlrp6 functions in the hematopoietic cell compartment rather than in nonhematopoietic cells [8]. Consistent with this observation, NLRP6 has been implicated in negatively regulating inflammatory responses in immune cells (discussed further below). In addition, *Nlrp6* mRNA expression is inducible such as by PPAR-γ agonists [33,38], and whether this occurs in hematopoietic cells in response to an as yet unidentified ligand to suppress tumorigenesis remains to be determined.

As a consequence of poor intestinal repair of DSS-induced epithelial damage, the epithelium barrier remains compromised in NLRP6-deficient mice, resulting in significantly elevated levels of proinflammatory, protumorigenic cytokines, and chemokines, likely in response to translocated bacteria [8]. Gene expression profiling of tumor and normal tissue in NLRP6-deficient and WT mice also demonstrated that NLRP6 is important for the negative regulation of factors involved in epithelial proliferation such as Wnt and Notch target genes [9]. Thus, although NRLP6-deficient mice exhibit increased inflammatory responses, consistent with a negative regulatory role in NF-κB and MAPK signaling as demonstrated in in vivo infection models [25], its role within the intestine is likely more related to the regulation of IL-18 production.

NLRP12 maintains intestinal homeostasis by negatively regulating inflammatory responses

Similar to NLRP6, NLRP12 also plays an important role in protecting against the development of DSS-induced colitis and inflammation-associated tumorigenesis with AOM/DSS [15, 16] (Fig. 1). In contrast to NLRP6, NLRP12-deficient mice are unable to transmit colitis susceptibility to WT mice after co-housing, suggesting that a mechanism distinct from microbiome regulation is responsible for its protective function [7]. Indeed, consistent with a role as a negative regulator of NF-κB and MAPK responses, NLRP12-deficient mice develop more severe colitis than WT mice. However, the precise mechanism by which NLRP12 negatively regulates NF-κB to protect against colitis and colitis-associated tumorigenesis remains to be determined as the two groups that reported this phenotype suggest disparate mechanisms [15, 16]. In both studies, the colitis in *Nlrp12*$^{-/-}$ mice is associated with significantly increased extent of disease, the production of proinflammatory cytokines and/or chemokines and the activation of NF-κB and ERK signaling in the colon [15, 16]. However, bone marrow chimeric experiments performed in the study by Zaki et al. [16] suggest that NLRP12 functions in the hematopoietic cell compartment to suppress tumorigenesis (Fig. 1). Moreover, the increased inflammatory responses are related to increased canonical and not noncanonical NF-κB activation. This is consistent with the observation that NLRP12-deficient BMDMs exhibit increased canonical, but not noncanonical, NF-κB and ERK activation in response to TLR ligands since excessive inflammatory responses to commensal bacteria in the setting of a breached epithelial barrier from DSS-induced colitis contributes to increased colitis and tumorigenesis. However, bone marrow chimeric experiments by Allen et al. [15] suggest that although NLRP12 function in both the hematopoietic and nonhematopoietic compartments is important for early disease manifestations of DSS-induced colitis, the nonhematopoietic compartment is ultimately important for limiting tumor numbers [15]. Furthermore, disease severity in NLRP12-deficient mice is predominantly associated with dysregulation of the noncanonical rather than canonical NF-κB pathway, consistent with previous studies by the same group demonstrating that NLRP12 interacts with NIK to regulate the p52 subunit of NF-κB [26]. The differences between studies can be partially reconciled by the fact that the study by Allen et al. also observed increased canonical NF-κB signaling in BMDCs after TLR ligand exposure in NLRP12-defiicent mice; moreover, noncanonical NF-κB signaling can influence both the canonical pathway and MAPK signaling [39–41]. The discrepancy in findings regarding whether NLRP12 is important in the hematopoietic or nonhematopoietic compartments is more difficult to explain. The analysis of NF-κB activation was in different cell types (BMDMs versus BMDCs), and there may have been differences in the colonies of *Nlrp12*$^{-/-}$ mice (e.g. the microbiota) that may have influenced the outcome of the bone marrow chimeric experiments. Regardless, taken together, these studies suggest an important role for NLRP12 in limiting inflammatory responses within the colon.

Conclusions

Exciting progress has been made in understanding how host immune receptors promote intestinal health. However, as demonstrated above, more than one mechanism may be important. Both NLRP6 and NLRP12 play protective roles in limiting the development of chemically-induced injury and carcinogenesis through regulation of IL-18 production, NF-κB and MAPK-dependent inflammatory responses, and prevention of dysbiosis. However, additional confirmatory experiments are needed to clarify the relative contributions of these different mechanisms in the regulation of intestinal homeostasis and to determine whether other mechanisms are involved. Discrepancies in the identity of the cell type(s) important for NLRP6 or NLRP12 function within the intestine and the differential impact of NLRP12 on canonical versus canonical NF-κB signaling remain. Moreover, our understanding of NRLP6 and NLRP12 function in intestinal homeostasis has been based entirely on one model of colitis involving DSS-induced epithelial injury. As additional mechanisms can be involved in the development of inflammatory bowel disease in humans, it would be important to also determine the effect of NLRP6 or NLRP12 deficiency in other models of colitis. A role for NLRP6 or NLRP12 in host defense against infections involving the intestine also remains to be investigated. Further advances in understanding the mechanism by which these two NLRs protect the intestine will be aided by the identification of the ligand or stimulus recognized by each NLR. This will also facilitate the development of therapeutics that modulate NLRP6 and NLRP12 function within the intestine to prevent or treat inflammation and cancer.

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Abbreviations: **AOM**: azoxymethane · **ASC**: apoptosis-associated speck-like protein · **BMDC**: bone marrow-derived DC · **BMDM**: bone marrow-derived macrophages · **CARD**: caspase recruitment domain · **DSS**: Dextran sulfate sodium · **LRR**: leucine-rich repeat · **NIK**: NF-κB-inducing kinase · **NLR**: Nod-like receptor · **NOD**: nucleotide-binding oligomerization · **PYD**: pyrin domain

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