# The role of pattern recognition receptors in intestinal inflammation

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**Recognition of microorganisms by pattern-recognition receptors (PRRs) is the primary component of innate immunity that is responsible for the maintenance of host–microbial interactions in intestinal mucosa. Dysregulation in host–commensal interactions has been implicated as the central pathogenesis of inflammatory bowel disease (IBD), which predisposes to developing colorectal cancer. Recent animal studies have begun to outline some unique physiology and pathology involving each PRR signaling in the intestine. The major roles played by PRRs in the gut appear to be the regulation of the number and the composition of commensal bacteria, epithelial proliferation, and mucosal permeability in response to epithelial injury. In addition, PRR signaling in lamina propria immune cells may be involved in induction of inflammation in response to invasion of pathogens. Because some PRR-deficient mice have shown variable susceptibility to colitis, the outcome of intestinal inflammation may be modified depending on PRR signaling in epithelial cells, immune cells, and the composition of commensal flora. Through recent findings in animal models of IBD, this review will discuss how abnormal PRR signaling may contribute to the pathogenesis of inflammation and inflammation-associated tumorigenesis in the intestine.**

## INTRODUCTION: THE ROLE OF TLRs AND NLRs IN HEALTHY GUT—PRRs AS REGULATORS OF INTESTINAL EPITHELIAL CELL (IEC) HOMEOSTATIS

The innate immunity provides a primary host response to microbial invasion, which induces an inflammatory nidus to localize the infection and prevent systemic dissemination of pathogens. The key process in this is the recognition of microbial agents by pattern-recognition receptors (PRRs). The PRRs include Toll-like receptors (TLRs), nucleotide binding oligomerization domain (NOD)-like receptors (NLRs), RNA helicases (RIG-I (retinoid acid-inducible gene-I), MDA5 (melanoma differentiation-associated gene 5), and LGP2 (laboratory of genetics and physiology gene 2)), C-type lectin receptors, and cytosolic DNA sensors (DNA-dependent activator of IFN-regulatory factors, absent in melanoma-2, interferon inducible protein 16 (leucine-rich repeat flightless-interacting protein 1), RNA polymerase III, DExD/H box RNA helicases, and IFI16), which sense evolutionarily conserved pathogen-associated molecular patterns (PAMPs) of microorganisms.[1] By detecting PAMPs, PRRs trigger sequential activation of intracellular signaling pathways leading to induction of a range of cytokines and chemokines that orchestrate the early host resistance to infection. Specifically, activation of NLRs results in the formation of a molecular scaffold complex (an inflammasome) that leads to the active release of interleukin (IL)-1β and IL-18 through caspase-1 activation (**Figure 1**). These PRRs signaling also facilitate the differentiation of T cells and B cells to establish antigen-specific adaptive immunity.[2–4]

Since the discovery of TLRs as a major family of PRRs, it has been of great interest whether or not they are functionally expressed in intestinal epithelial interface and what roles they have in the gastrointestinal tract. Because of the unique nature of the gut where diverse microorganisms coexist, microbial-sensing TLRs may have special roles in mucosal homeostasis. Among the 13 TLRs discovered, TLR1 through TLR9 have been identified as being expressed in human IECs.[5,6] However, the functional consequences of these TLRs in healthy gut physiology have yet to be fully determined.

Although it has not been fully understood how the TLR responses in IECs are regulated in the face of commensal

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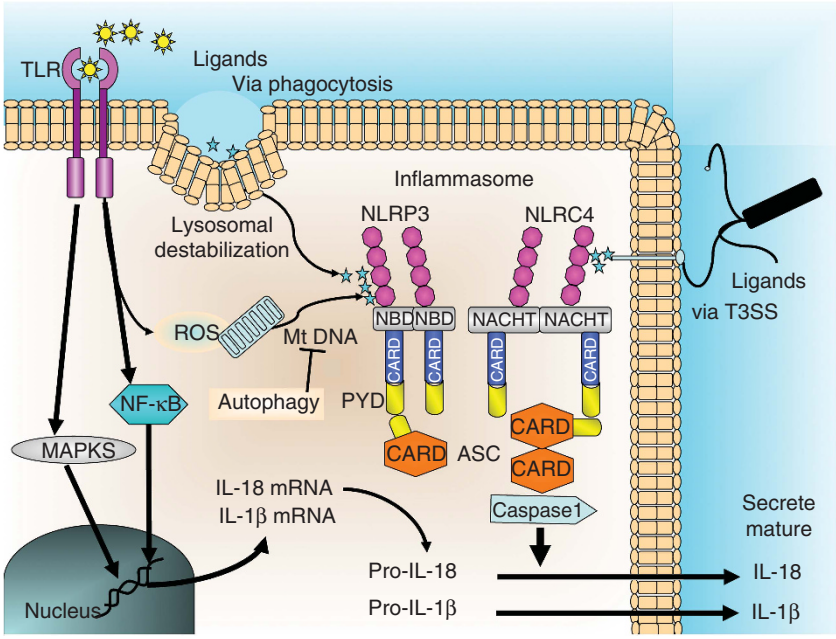


**Figure 1** The pattern-recognition receptor pathway inducing production of mature interleukin (IL)-18 and IL-1β. Upon recognition of pathogens, Toll-like receptor (TLR) signaling (mainly TLR4) induces transcription of IL-18 and IL-1β. Expression of mRNA for these genes produces pro-forms of IL-18 and IL-1β protein within the cytosol. At the same time, TLR signaling induces reactive oxygen species (ROS) generation and following release of mitochondrial DNA, which activates nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). NLRs are also activated by detecting various ligands introduced via phagocytosis followed by lysosomal stabilization, bacterial type III secretion system, or intracellular metabolic and/or enzymatic activities. The activated NLRs are homo- or hetero-oligomalized to form multi-protein complex termed "inflammasome" that activate caspase-1. Activated caspase-1 induces proteolytic conversion of pro-IL-18 and pro-IL-1β to mature IL-18 and IL-1β to release the active form of these cytokines. ASC, apoptosis-associated speck-like protein; CARD, caspase activation and recruitment domain; CIITA, MHC class II transcription activator; HET-E, incompatibility locus protein from podospora anserina; MAPK, mitogen-activated protein kinase; Mt DNA, mitochondrial DNA; NACHT, neuronal apoptosis inhibitory protein; NBD, nucleotide-binding domain; NF, nuclear factor; NLRP3, NLR protein 3; PYD, pyrin domain; TP1, telomerase-associated protein.

bacteria in the human intestine, *in vitro* data has demonstrated hyporesponsiveness of IECs to TLR2 and TLR4 ligands.[5,6] The underlying mechanism of this observation comprises a decrease in TLR surface expression and the induction of an inhibitory molecule of TLR signaling after ligand stimulation. Antigen-presenting cells in the lamina propria also appear to be unresponsive to TLR ligands.[7] Other TLRs are normally expressed in endosomes (TLR3, TLR7 to TLR9) or basolateral membrane (TLR5), where these TLRs are not exposed to pathogens unless pathogens get into the cells or invade mucosa. NOD and NLRs are also expressed in the cytoplasm and thus do not recognize extracellular pathogens unless pathogens inject the cells' effector proteins (**Table 1**). On the other hand, constitutive activation of selected TLR signaling in IECs may be required for epithelial homeostasis. IEC-specific deletion of the downstream molecules of TLR signaling such as myeloid differentiation primary response gene 88 (MyD88), transforming growth factor-β-activated kinase 1, and nuclear factor-κB essential modulator in mice results in spontaneous intestinal inflammation.[8–10] TLR9 can be expressed on the apical or the basolateral sides of IECs, in which ligand recognition activates a tolerogenic or inflammatory responses, respectively.[11] In addition to IECs, many TLRs are expressed and have unique roles in Paneth cells, goblet cells, and enteroendocrine cells in the intestine.[12–14] These findings highlight a unique feature of PRRs in IECs that establishes selective responses and tolerance to the commensal flora at the mucosal interface.

Functionally, epithelial PRRs contribute to balancing the composition of luminal microorganisms by regulating the secretion of a range of antimicrobial peptides and mucosal immunoglobulin A (IgA). Mice deficient in MyD88 have demonstrated a significant defect in production of multiple antimicrobial peptides in Paneth cells, resulting in increased bacterial penetration to the mesenteric lymph nodes.[12] TLR9$^{-/-}$ and NOD2$^{-/-}$ mice have impaired expression of Paneth cell cryptdin (mouse α-defensin) compared with wild-type (WT) mice.[11,15] Patients with Crohn's disease (a chronic intestinal inflammatory condition) who carry NOD2 mutations have defective production of α-defensins (HD5, HD6) by Paneth cells along with an increased abundance of mucosa-associated bacteria.[16,17] In addition, signaling through TLR2, TLR3, TLR4, NOD1, NOD2, and NLR Protein 3 (NLRP3) have all been implicated with the expression of β-defensins in IECs.[18,19] Most TLR signaling in IECs induces B cell-activating factors that lead to immunoglobulin class switch recombination in lamina propria B cells without T-cell activation, resulting in IgA secretion.[20,21] In addition, activation of TLR3 and TLR4 has been shown to induce the expression of polymeric immunoglobulin receptor, an epithelial immunoglobulin transporter, by IECs that enhances luminal IgA secretion.[22,23] Therefore, TLR signaling in IECs

**Table 1 Expression of PRRs in IECs**

| PRRs | Expression in IECs Human/mouse[126–132] | Expression pattern | Ligands | Sources |
|---|---|---|---|---|
| *TLRs*[5,6] | | | | |
| TLR1 | +/ND | Surface membrane | Triacyl lipoproteins | Mycobacteria |
| TLR2 | +/+ | Surface membrane | Peptidoglycan, lipoteichoic acid | Gram-positive bacteria |
| TLR3 | +/+ | Endosomal membrane | Double-stranded RNA, poly(I:C) | Viruses |
| TLR4 | +/+ | Basolateral and endosomal membrane | Lipopolysaccharide | Gram-negative bacteria |
| TLR5 | +/+ | Basolateral membrane | Flagellin | Bacteria |
| TLR6 | +/ND | Surface membrane | Diacyl lipopeptides Zymosan | Mycobacteria fungi |
| TLR7 | +/ND | Endosomal membrane | Single-stranded RNA | Viruses |
| TLR8 | +/ND | Endosomal membrane | Single-stranded RNA | Viruses |
| TLR9 | +/+ | Endosomal membrane | CpG-ODN | Bacteria and viruses |
| *NLRs*[133] | | | | |
| NOD1 | +/+ | Cytoplasm | Meso-lanthionine, meso-DAP | Bacteria |
| NOD2 | +/+ (inducible) | Paneth cell cytoplasm (inducible in IECs) | Muramyldipeptide | Bacteria |
| NLRC4[114] | ND | Cytoplasm | Flagellin, etc. | Bacteria |
| NLRP3[82] NLRP6[27,62] | +/+ | Cytoplasm | Muramyldipeptide, Bacterial RNA, crystals, mitcondoria DNA | Bacteria, viruses, uric acid crystals maitotoxin |
| *RNA helicases*[1] | | | | |
| RIG-I | +/+ | Cytoplasm | Cytoplasmic dsRNA | Viruses |
| MDA5 | +/+ | Cytoplasm | Cytoplasmic dsRNA | Viruses |
| *C-type lectin*[134–139] | | | | |
| CL-P1 | ND | Surface membrane | D-galactose, L- and D-fucose, GalNAc, T and Tn antigens | Bacteria, fungi |
| Monocyte mannose receptor | ND | Surface membrane | Galactose/*N*-acetylgalactosamine | Fungi |
| Mannose-binding lectin | ND | Surface membrane | Mannose and/or *N*-acetylglucosamine | Bacteria |
| Ficolins | ND | Surface membrane | *N*-acetyl-D-glucosamine | Bacteria |
| DC-SIGN | ND | Surface membrane | High-mannose-containing glycoproteins | Virus, bacteria, fungi |
| Dectins | +/ND | Surface membrane | β-glucans | Fungi |
| *Other DNA sensors*[1] | | | | |
| DAI | ND | Cytoplasm | DNA | Virus |
| AIM-2 | ND | Cytoplasm | DNA | Virus |
| RNA Polymerase III | ND | Cytoplasm | DNA | Virus |
| IFI16 | ND | Cytoplasm | DNA | Virus |

AIM-2, absent in melanoma-2; CpG-ODN, cytosine-phosphate-guanosine oligodeoxynucleotides; DAI, DNA-dependent activator of IFN-regulatory factors; DC-SIGN, dendritic cell–specific intercellular adhesion molecule-3-grabbing non-integrin; IEC, intestinal epithelial cell; IFI16, interferon inducible protein 16; MDA5, melanoma differentiation-associated gene 5; ND, not detected; NLR, nucleotide-binding oligomerization domain (NOD)-like receptor; NLRP, NLR protein; poly(I:C), polyinosinic-polycytidylic acid; PRR, pattern-recognition receptor; RIG-I, retinoid acid-inducible gene-I; TLR, Toll-like receptor.

is involved in multiple steps in intestinal IgA secretion. The antimicrobial peptides and the secretory IgA balance microbial composition, limiting penetration of commensal bacteria in the intestine.[24] Therefore, regulation of commensal bacterial burden and composition is likely to be a major function of PRRs to maintain intestinal homeostasis.

## TLRs AND NLRs IN GUT INFLAMMATION—WHEN MICROBE SENSING GOES AWRY

Targeted PRR gene knockout mice have provided important information regarding intestinal phenotypes of individual PRRs (**Table 2**). Despite their importance in regulation of commensal flora, only mice that are deficient either in TLR5, NLRP6, or RIG-I have shown to develop spontaneous intestinal inflammation.[25–27] The tolerogenic nature of PRRs at the intestinal host–microbe interface may allow the absence of PRR signaling to keep normal intestinal integrity. Nevertheless, the lack of spontaneous phenotypes in most PRR-deficient mice suggests the possible involvement of compensatory mechanisms. As many pathogens carry multiple PAMPs, a pathogen can be redundantly recognized by multiple PRRs within a single cell of the host. Because PRRs share several key innate immune signaling pathways, intestinal immune homeostasis may be preserved by providing a sufficient host response to commensal microorganisms in most PRR-deficient mice (**Figure 2**). However, many PRR-deficient mice develop more severe intestinal inflammation than WT mice in the setting of epithelial injury or abnormal cytokine environments such as the absence of IL-10, highlighting the requirement of proper PRR responses for danger signaling during pathological conditions.

### The role of TLRs in intestinal inflammation

The role of each TLR signaling in the development of intestinal inflammation may vary depending on the cell types in mucosa and interactions between individual TLR signaling. The spontaneous intestinal inflammation in TLR5$^{-/-}$ mice implies a distinct role of TLR5 in maintenance of mucosal immune response to commensal bacteria. Induction of colitis in TLR5$^{-/-}$ mice is inhibited by cross breeding these mice with TLR4$^{-/-}$ mice, indicating that the mucosal inflammation is TLR4-dependent without TLR5 regulation.[25] It is still unclear which cell types in intestinal mucosa are responsible for the TLR4-mediated induction of colitis. However, epithelial TLR4 signaling is unlikely to be involved, because constitutively active expression of epithelial TLR4 does not induce mucosal inflammation in the villin-TLR4 transgenic mice.[21,28] Though, selective deletion of MyD88 in IECs results in spontaneous inflammation of the small intestine,[8] MyD88 signaling in myeloid cells is required for the development of intestinal inflammation during colonization of RAG2$^{-/-}$ mice with *Helicobacter hepaticus*.[29] Consistently, TLR4 signaling in myeloid cells has been implicated with mucosal inflammation during enteric *Citrobacter rodentium* infection.[30] Therefore, TLR signaling in the myeloid cell compartment has more

**Table 2 Intestinal phenotypes and their susceptibility to colitis in PRR knockout mice**

| PRR target | Gene modification | Intestinal phenotypes |
|---|---|---|
| *TLRs* | | |
| TLR2[49,52] | Knockout | Increased susceptibility to DSS colitis |
| TLR4[39,49,50] | Knockout | Increased susceptibility to DSS colitis. Exacerbate or ameliorate IL-10$^{-/-}$ colitis (depending on commensals or *Helicobacter hepaticus*) |
| TLR5[a,25,43] | Knockout | Spontaneous colitis (25%), increased susceptibility to DSS colitis |
| TLR9[60,140] | Knockout | Increased susceptibility to DSS acute colitis but resistant to chronic DSS colitis |
| *NLRs* | | |
| NOD1[72,73] | Knockout | Increased susceptibility to DSS colitis |
| NOD2[15,68,69,71] | Knockout | Increased susceptibility to DSS colitis and TNBS colitis. Defective cryptdin expression |
| | Knock-in of human NOD2-3020insC mutation | Increased susceptibility to bacterial-induced intestinal inflammation |
| NLRC4[114] | Knockout | Similar susceptibility to DSS colitis as WT mice |
| NLRP3[74–77,81,82] | Knockout | Increased or reduced susceptibility to DSS colitis (depending on the reports). Increased or reduced susceptibility to TNBS colitis |
| NLRP6[a,27,61,62] | Knockout | Spontaneous colitis (crypt hyperplasia and inflammatory cell recruitment), increased susceptibility to DSS colitis |
| *RNA helicases* | | |
| RIG-I[a,26] | Knockout | Spontaneous intestinal inflammation |

DSS, dextran sulfate sodium; IL, interleukin; NLR, nucleotide-binding oligomerization domain (NOD)-like receptor; NLRP, NLR protein; PRR, pattern-recognition receptor; RIG-I, retinoid acid-inducible gene-I; TLR, Toll-like receptor; TNBS, 2,4,6-trinitrobenzenesulfonic acid; WT, wild type.
[a]Spontaneous phenotype.

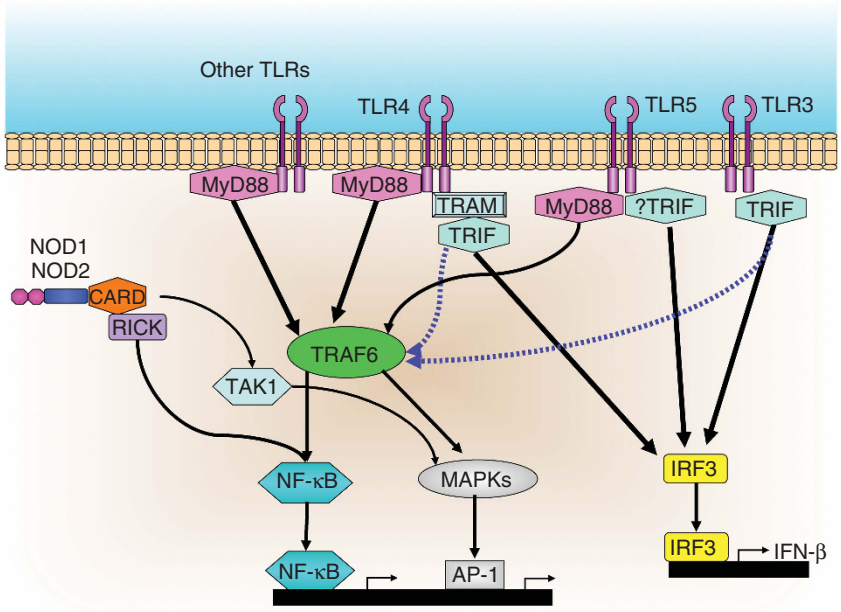


**Figure 2** Pattern-recognition receptors share immune signaling pathways. Most Toll-like receptors (TLRs) except for TLR3 induce nuclear factor (NF)-κB activation through the myeloid differentiation primary response gene 88 (MyD88) pathway. TLR3 exclusively induces IRF3 activation through the TIR-domain-containing adapter-inducing interferon (IFN)-β (TRIF) pathway. The TRIF pathway is shared by TLR4 and TLR5 (in intestinal epithelial cells). Nucleotide-binding oligomerization domain 1 (NOD1) and NOD2 also activate NF-κB via the RICK (receptor-interacting serine/threonine kinase) pathway. TLRs and NODs also induce activate mitogen-activated protein (MAP)-kinases. AP-1, activator protein 1; CARD, caspase activation and recruitment domain; IRF, interferon regulatory factor; TAK1, transforming growth factor-β-activated kinase 1; TRAF, tumor necrosis factor receptor-associated factor; TRAM, TRIF-related adapter molecule.

significant roles than epithelial TLR signaling in induction of mucosal inflammation in the intestine.

Dysbiosis of intestinal microbiota has been implicated as a prerequisite for the development of inflammatory bowel disease (IBD).[31] Given the importance of mucosal PRR signaling in the maintenance of commensal flora in the normal intestine, PRR deficiency may alter the composition of commensal flora that may be involved in the pathogenesis of colitis. The colitic TLR5$^{-/-}$ mice show increased intestinal bacterial burden and variability of commensal flora compared with non-colitic TLR5$^{-/-}$ mice.[25] An increase of the *Enterobacteriaceae* composition has been shown during chemically induced colitis in mice, which correlates with the severity of intestinal inflammation.[32] The extent of this shift in commensal composition during colitis is less in TLR2/TLR4 double-deficinet mice than WT mice.[32] In addition, the TLR2/TLR4 double-deficient mice have significantly less burdens of *Bacteroides*, *Prevotella*, and *Enterococcous* species in the intestine compared with WT mice.[32] However, the alteration of composition of commensal flora in TLR-deficient mice is limited at baseline and mostly dependent on maternal transmission rather than TLR regulation of host–commensal interactions.[33] IL-10$^{-/-}$ mouse is a well-established model of colitis as they develop spontaneous colitis due to uncontrolled pro-inflammatory cytokine production in response to commensal flora.[34] In fact, different types of commensal bacteria cause intestinal inflammation with different onset, location, and degree in IL-10$^{-/-}$ mice.[35] Interestingly, nullifying MyD88 in IL-10$^{-/-}$ mice results in the prevention of colitis induction.[36] There are conflicting reports regarding inhibition or acceleration of colitis in TLR4/IL-10 double-knockout mice, which may be explained by differences in flora across various facilities.[37–39] Cross breeding IL-10$^{-/-}$ mice with TLR2$^{-/-}$ mice have shown exacerbation of colitis, but crossing IL-10$^{-/-}$ mice with TLR9$^{-/-}$ mice did not alter intestinal phenotype of IL-10$^{-/-}$ mice despite both TLR2 and TLR9 signal through MyD88.[37,40] Nullifying MyD88 in other commensal-dependent spontaneous colitis models, such as IL-2$^{-/-}$ mice or Tbx21$^{-/-}$Rag$^{-/-}$ mice both show similar severity of colitis as their MyD88-sufficient counterparts.[36,41,42] Interestingly, treatment of TLR4/TLR5 double-knockout mice with IL-10 receptor neutralizing antibody (IL-10R mAb) results in development of colitis but IL-1R-deficient TLR5$^{-/-}$ mice does not show signs of colitis in response to IL-10R mAb treatment.[43] Because IL-1R shares MyD88 signaling with other TLRs, these results indicate that IL-1 and MyD88 signaling may have a role for induction of colitis seen in TLR5$^{-/-}$ mice, although in another study IL-1R$^{-/-}$ mice displayed worse colitis induced by dextran sulfate sodium (DSS).[44] These conflicting results suggest that the role of MyD88 in comensal dependent colitis may differ depending on its upstream inputs and cytokine profile.

Demand for PRR signaling becomes higher in the setting of epithelial damage than homeostatic conditions in the gut. Regardless of the cause, disruption of the epithelial barrier integrity leads to exposure of mucosal immune system to the mass of commensals. During epithelial damage, PRRs also recognize damage-associated molecular patterns that are

released from host tissues.[45,46] Mouse DSS-induced colitis is a well-established colitis model specifically relevant to investigate intestinal innate immune responses to epithelial injury as this model is induced by chemical damage of the epithelium and does not require adaptive immunity.[47] In the DSS-induced colitis, it has been shown that germ-free mice demonstrate worse colitis compared with conventional mice, but similar severity of colitis has been noted between conventional mice and the commensal-depleted mice that are orally supplemented with TLR2 and TLR4 ligands.[48,49] Consistently, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice show increased severity of DSS-induced colitis.[49,50] MyD88, but not TIR-domain-containing adapter-inducing interferon-β (TRIF)-deficient, mice also demonstrated more severe disease than WT mice in this model, suggesting the importance of MyD88-dependent TLR signaling in protection against mucosal damage.[50,51] However, the underlying mechanisms of protection differ between TLR2 and TLR4. TLR2 signaling in IECs induces tight junctional protein ZO-1 through activation of protein kinase C, which strengthen epithelial barrier integrity and resists cell apoptosis.[52,53] TLR2 signaling also induces cytoprotective trefoil factor production.[54] By contrast, TLR4-mediated MyD88 signaling in IECs and subepithelial macrophages induces cyclooxygenase 2 (COX-2) that amplifies mucosal prostaglandin $E_2$ ($PGE_2$) synthesis, supporting epithelial survival.[55–58] TLR4 signaling in IECs also induces expression and release of amphyregulin and epiregulin that activate epidermal growth factor receptor.[59] Therefore, multiple cytoprotective functions in IECs are covered by several TLRs signaling, but defects in different aspects of the cytoprotection may result in similar manifestations in the intestine.

The DSS colitis model may rely on different mechanisms to induce acute vs. chronic inflammation. For example, TLR9$^{-/-}$ mice have shown to be more susceptible to acute DSS colitis, but they demonstrate less severe manifestations of colitis during repeated cycles of DSS treatments compared with WT mice.[11,60] Suppression of TLR9 signaling by adenoviral oligodeoxynucleotides, which is known to block the effect of bacterial cytosine-phosphate-guanosine oligodeoxynucleotides, have demonstrated a suppressive effect of intestinal inflammation in various mouse models of chronic colitis.[60] Therefore, bacterial DNA from commensal flora is one of the factors inducing intestinal inflammation through activation of TLR9 during chronic colitis. These results indicate that TLR signaling during DSS colitis contribute to cytoprotection and mucosal restitution, but at the same time, it may be involved in sustained mucosal inflammation in response to commensal bacteria.

### The role of NLRs in intestinal inflammation

Intestinal inflammation in NLR-deficient mice has been extensively studied (**Table 2**). The most striking phenotype was observed in NLRP6$^{-/-}$ mice.[27] Mice deficient in NLRP6 and its signaling adapter apoptosis-associated speck-like protein (ASC) show increased number of CD45$^{+}$ cells in lamina propria, crypt hyperplasia in the colon and enlargement of Peyer's patches with formation of germinal centers.[27] These mice have increased mucosal permeability during DSS colitis and are unable to recover from colitis.[61,62] Bone marrow chimera experiments demonstrate that NLRP6 deficiency in non-hematopoietic cells is responsible for the increased susceptibility to DSS colitis.[27] Among bone marrow chimera mice developed by WT mice and NLRP6$^{-/-}$ mice, mucosal IL-18 production in response to DSS treatment is significantly reduced only when NLRP6$^{-/-}$ mice are used as recipient.[27] Similar increase in DSS susceptibility is observed when WT bone marow is transferred to IL-18$^{-/-}$ mice but not IL-1β$^{-/-}$ mice, indicating that NLRP6-mediated IL-18 production from intestinal non-hematopoietic cells contributes to the resistance against DSS-induced colitis.[27] As the expression of NLRP6 in IECs is transcriptionally regulated by nuclear receptor peroxisome proliferator-activated receptor γ which is known to be induced by TLR4 signaling and has potent anti-inflammatory properties, NLRP6-mediated mucosal protection against DSS-induced injury involves epithelial response to commensal bacteria through TLR4 signaling.[63–65] Furthermore, NLRP6$^{-/-}$ mice have significantly elevated expression of C-C motif chemokine ligand 5 (CCL5) chemokine in the colon compared with WT mice, suggesting that NLRP6 has an important role for the negative regulation of CCL5 to maintain intestinal host–commensal homeostasis.[27] Interestingly, the susceptibility to intestinal inflammation and upregulation of CCL5 in NLRP6$^{-/-}$ mice is associated with alterations of commensal composition (especially high prevalence of genus *Prevotellaceae* and group TM7 bacteria).[27] Co-housing with NLRP6$^{-/-}$ mice and hence adopting similar flora, WT mice experienced upregulation of CCL5 in the colon. Although this upregulation of intestinal CCL5 did not cause spontaneous intestinal inflammation in WT mice, these mice had worse colitis in response to DSS than WT mice that are housed by themselves. Increased susceptibility to DSS-induced colitis by co-housing with NLRP6$^{-/-}$ mice was not observed in CCL5$^{-/-}$ mice confirming CCL5 as an effector molecule in exacerbation of colitis by the commensal alteration. Although IL-18$^{-/-}$ mice have elevated CCL5 levels in the intestine, IL-18$^{-/-}$ mice carry distinct commensal bacteria from that in NLRP6$^{-/-}$ mice.[27] Commensal bacteria-dependent increase of colitis susceptibility has also been observed in Tbx21$^{-/-}$Rag$^{-/-}$-deficient mice and angiotensin-converting enzyme 2 (ACE2)-deficient mice.[66,67] Therefore, IL-18-dependent and -independent mechanisms or multiple types of commensal bacteria may be involved in the intestinal phenotype of NLRP6$^{-/-}$ mice.

As NOD2 gene mutations are associated with Crohn's disease, mice with genetically modified *Nod2* gene have been engineered and studied. Although NOD2-knockout mice (NOD2$^{-/-}$ mice) do not develop spontaneous colitis, these mice have increased burdens of *Bacteroides*, *Firmicutes*, and *Bacillus* species in ileal mucosa compared with WT mice and are more susceptible to 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis.[68,69] Bone marrow chimera model demonstrated that NOD2 deficiency in hematopoietic cells increases severity of TNBS-induced colitis.[68] Furthermore,

transgenic mice that overexpress NOD2 under major histocompatibility complex class II promoter (antigen-presenting cells) demonstrate increased resistance to TNBS-induced colitis.[70] Mice carrying the *Nod2* mutation (*Nod2*$^{2939iC}$ mice), noted in patients with Crohn's disease (3020insC), have worse colitis secondary to DSS treatment compared with controls.[71] Although this mouse strain is recently found to have a duplication of the 3′ end of the WT *Nod2* locus, the altered function of NOD2 with this mutation that augments IL-1β production has been validated (http://www.sciencemag.org/content/333/6040/288.3.full.pdf). In addition, NOD1$^{-/-}$ mice and Nod1/Nod2 double-knockout mice have been reported to be more susceptible to DSS-induced colitis compared with control mice partly due to increased mucosal permiability during colitis.[72,73] NOD2 has shown to regulate intestinal commensal flora through the secretion of bacteria-killing factors.[69] Therefore, impaired NOD2 signaling alters commensal composition and increases the susceptibility to intestinal inflammation that may be associated with defective secretion of antimicrobial peptides in the intestine and abnormal immune cell response to the altered commensal flora. The increased mucosal permeability may facilitate mucosal immune cell exposure to luminal contents.

The role of NLRP3 in colitis has been studied in several mouse colitis models with various results. Multiple reports have demonstrated that mice deficient in NLRP3 or its downstream signaling molecules ASC and caspase-1 have increased severity of DSS-induced colitis compared with WT mice.[74–77] NLRP3$^{-/-}$ mice are also more susceptible to TNBS-induced colitis than WT mice.[74,75] Bone marrow chimera experiments highlight the importance of NLRP3 in intestinal stromal cells rather than hematopoietic cells in resistance to DSS-induced colitis.[75] Both NLRP3$^{-/-}$ mice and caspase-1$^{-/-}$ mice show increased mucosal permeability accompanied by impaired epithelial proliferation during DSS colitis.[75] The number of proliferating epithelial cells and mucosal permeability in these mice are similar to WT mice at baseline, indicating that these mice have defective repair responses to mucosal injury. As intraperitoneal injection of recombinant IL-18 during DSS treatment reduced colitis severity in caspase-1$^{-/-}$ mice, NLRP3-mediated protection from DSS-induced mucosal injury might be mediated by IL-18 production through caspase-1 activation.

Earlier studies, however, have shown that blocking IL-18 hampers mucosal induction of pro-inflammatory cytokines and chemokines and attenuates severity of DSS-induced colitis in mice.[78,79] IL-18 transgenic mice exhibit an increased susceptibility to DSS-induced colitis.[80] The other report has described that absence of caspase-1 protects mice from DSS-induced colitis especially in chronic phase.[78] Finally, two reports have demonstrated that NLRP3$^{-/-}$ mice develop a less severe intestinal inflammation during DSS-induced colitis and that pharmacological inhibition of caspase-1 attenuated DSS-induced colitis.[27,81]

There appears to be two phenotypes of NLRP3 in colitis. Worse DDS-induced colitis in NLRP3$^{-/-}$ and caspase-1$^{-/-}$ mice are likely due to impaired epithelial proliferation after epithelial injury.[75,77] The precise mechanism by which NLRP3 regulates epithelial proliferation is still obscure, though this possibly involves interferon (IFN)-γ and IL-18 in regulation of cell proliferation.[82–84] By contrast, NLRP3$^{-/-}$ mice have reduced mucosal expression of IFN-γ and impaired neutrophil activity during DSS-induced colitis, which may attenuate intestinal inflammation in this model.[74,85] IL-18 is known to induce IFN-γ production directly by natural killer cells and synergistically with IL-12 by T cells.[84,86] Therefore, NLRP3 may have both promotive and suppressive role in DSS-induced colitis.

Which of these opposite NLRP3 phenotype dominates may be dictated by the composition of the intestinal flora. As demonstrated by experiments of WT mice co-housed with NLPR6$^{-/-}$ mice, severity of DSS-induced colitis can be substantially influenced by the composition of commensal flora.[27] NLRP3$^{-/-}$ mice have shown a unique expression profile of intestinal antimicrobial peptides and distinct intestinal microbiota from WT mice.[74] As development of intestinal commensal flora depends on maternal gut flora and immunity that differ between breeding facilities, it is possible that mice raised in different facilities have distinct flora profiles that lack some of the key populations responsible for each NLRP3-associated intestinal phenotype.[32,87,88]

NLR inflammasome is activated in cells of intestinal mucosa during colitis. DSS has been shown to activate NLRP3 inflammasome and releases IL-1β from lipopolysaccharide-stimulated macrophages through potassium efflux, lysosomal maturation, and production of reactive oxygen species.[81] Effective cytokine release by inflammasome requires TLR signaling that induces precursors of IL-1β and IL-18 (**Figure 1**). Commensal bacteria through TLR signaling may individually activate NLR inflammasomes by inducing adenosine triphosphate (ATP), lysosomal leakage, and production of reactive oxygen species.[89–91] Recently, we have described that oxidized mitochondrial DNA induced by the above stimuli binds to NLRP3 and induces inflammasome activation during cell apoptosis, but this is inhibited by autophagy activation.[92] Therefore, host–microbial interactions in intestinal mucosal interface are orchestrated by TLRs and NLRs through regulation of the balance between autophagy and inflammasome activation.

## TLRs AND NLRs IN INFLAMMATION-ASSOCIATED COLORECTAL CANCER

Chronic intestinal inflammation has long been suggested to trigger tissue neoplastic transformation as higher incidence of intestinal cancer has been observed in patients with IBD. Extensive studies have identified the molecular pathogenesis of sporadic colon cancer based on genetic alterations. Although the process of developing colitis-associated cancer involves some of these genetic abnormalities, unique aspects of colon carcinogenesis in the setting of chronic colitis have been illuminated.[93,94] As inflamed intestinal cells in patients with IBD have these genetic alterations before developing

histological features of dysplasia, genetic alterations in colitis-associated cancer may be a secondary step rather than primary cause of tumorigenesis.[95] It is likely that abnormal signaling in some PRRs leads to uncontrolled expression of the genes and enzymes regulating cell proliferation, apoptosis, and DNA repair before the gene alterations. Although precise mechanisms underlying initiation and/or promotion of the inflammation-associated intestinal cancer have yet to be fully determined, frequent cycles of injury and repair of the epithelium in the presence of tumorigenic cytokines, chemokines, and prostaglandins may predispose to genetic mutations, which increases neoplastic risk.[96,97] As conventionalization of germ-free animals accelerates epithelial proliferation in the intestine, PRR signaling may have an important role in regulation of epithelial proliferation.[98] It has been reported that TLR-mediated MyD88 signaling in subepithelial macrophages regulates crypt stem cell differentiation and epithelial proliferation through expression of COX-2 and $PGE_2$.[57,58] TLR4 stimulation has also been shown to induce proliferation of human IECs via induction of epidermal growth factor receptor ligands.[99,100] The inflammatory milieu may enhance surface expression of TLR2 and TLR4, leading to IECs responsiveness to their ligands.[101,102] These results suggest that abnormal TLR signaling, especially TLR2 and TLR4 in both IECs and subepithelial macrophages, may induce aberrant epithelial proliferation and thus may contribute to cancer development in the setting of chronic inflammation.

Recent reports regarding PRR phenotypes in mouse models of colitis-associated (AOM-DSS) and spontaneous multiple intestinal (Apc/Min) neoplasia appear to show some important pathways in the regulation of intestinal tumorigenesis (**Table 3**). The AOM-DSS (a single injection of azoxymethane followed by repeated DSS treatment and recovery) model mimics human colitis-associated cancer as it represents repeated mucosal injury and repair, leading to epithelial proliferation and dysplastic transformation in the colon.[103] In this model, $TLR4^{-/-}$ mice are protected from tumor development due to decreased expression of mucosal COX-2, $PGE_2$, and amphiregulin.[59] Supplementation of $PGE_2$ during the recovery of colitis bypasses the protective phenotype of $TLR4^{-/-}$ mice against intestinal tumors along with sustained upregulation of COX-2 in subepithelial macrophages and epithelial amphiregulin production.[56] Therefore, the upregulation of $PGE_2$ during the recovery phase of colitis is a key for colitis-associated tumorigenesis involved in TLR4 signaling. TLR4 expression only in stroma but not in myeloid cells restored tumor incidence in $TLR4^{-/-}$ mice.[104] In addition, transgenic expression of constitutively active TLR4 results in increased mucosal $PGE_2$ production and tumor development in this model.[28] These results indicate that epithelial TLR4 signaling contribute to tumor development through mucosal production of $PGE_2$ in the setting of chronic colitis.

On the other hand, $TLR2^{-/-}$ mice have demonstrated increased tumor development in the AOM-DSS model.[105] Although IECs in $TLR2^{-/-}$ mice are less proliferative and more apoptotic in normal mucosa, they become more proliferative and less apoptotic during chronic colitis compared with WT mice.[105] Underlying mechanism of increased tumor burden in $TLR2^{-/-}$ mice is associated with strong activation of epithelial STAT3 (signal transducer and activator of transcription 3) and higher expression of tumorigenic cytokines (IL-6, tumor necrosis factor-α (TNF-α), and IL-17A) in intestinal mucosa. Mucosal TNF-α signaling and IL-6-mediated STAT3 activation are known to be indispensable during tumor development in this model.[106] In addition, STAT3-mediated T helper type 17 cell generation has been shown to facilitate tumor development in Apc/Min mice.[107]

**Table 3 Susceptibility to colitis-associated tumor in PRR knockout mice**

| PRR knockouts | Susceptibility | Possible pathogenesis |
|---|---|---|
| *TLRs* | | |
| TLR2[105] | Increased | Increased epithelial proliferation due to greater expression of tumorigenic cytokines and epithelial STAT3 activation |
| TLR4[56,59,104] | Decreased | Defective mucosal expression of COX-2 and following production of $PGE_2$. Defective mucosal production of EGFR ligand, amphiregulin |
| *NLRs* | | |
| NOD1[72] | Increased | Increased intestinal inflammation and greater expression of tumorigenic cytokines resulting in epithelial proliferation |
| NOD2[117] | Increased | Transmissible by co-housing (The pathogeneis involves commensal bacteria) |
| NLRC4[114] | Increased/the other report shows similar susceptibility to WT mice | Defective caspase-1 activation resulting in protection of epithelial cells from apoptosis |
| NLRP3[76] | Increased | Defective caspase-1 activation. Defective mucosal IL-18 release |
| NLRP6[61,62] | Increased | Increased expression of Wnt-target genes. Defective mucosal IL-18 release |
| NLRP12[115,116] | Increased | Increased intestinal inflammation and greater expression of tumorigenic cytokines. Increased epithelial proliferation |

COX-2, cyclooxygenase 2; EGFR, epidermal growth factor receptor; IL, interleukin; NLR, nucleotide-binding oligomerization domain (NOD)-like receptor; NLRP, NLR protein; $PGE_2$, prostaglandin $E_2$; PRR, pattern-recognition receptor; STAT3, signal transducer and activator of transcription factor 3; TLR, Toll-like receptor; WT, wild type.

It has been reported that increased expression of these cytokines in TLR2$^{-/-}$ mice is due to compensatory activation of TLR4.[30,108] Therefore, enhanced tumorigenesis in TLR2$^{-/-}$ mice may involve TLR4 signaling.

MyD88$^{-/-}$ mice have demonstrated multiple phenotypes in the development of intestinal tumors. MyD88$^{-/-}$ mice develop more tumors in the AOM-DSS model than WT controls but fewer tumors in AOM-treated IL-10$^{-/-}$ model or AOM-oxazolone model compared with their WT counterparts.[109–111] This discrepancy is likely due to differences in inflammation between the models, as MyD88 deficiency results in increased severity of colitis in the AOM-DSS model but reduced colitis in AOM-treated IL-10$^{-/-}$ model and no effect on colitis severity in the AOM-oxazolone model.[109–111] It is important to note that there is no increase in IEC proliferation after AOM-DSS treatment in MyD88$^{-/-}$ mice.[109] The underlying mechanism of the increased susceptibility of MyD88$^{-/-}$ mice to the AOM-DSS induced intestinal tumor is upregulation of the genes associated with Wnt signaling, DNA repair, and angiogenesis.[109] In addition, MyD88$^{-/-}$ mice showed more frequent clonal mutations in the β-catenin gene in IECs during AOM-DSS treatment.[109] Without chronic inflammation, MyD88 deficiency results in resistance to intestinal tumor development in the Apc/Min and AOM (without DSS) models.[109,112] Therefore, MyD88 signaling may act both in a tumorigenic and anti-tumorigenic capacity depending on the presence and the types of chronic inflammation in the intestine. This is possible because MyD88 transduces multiple receptor signaling pathways, including most TLRs, IL-1R, and IL-18R, which individually lead to the induction of a distinct set of genes.[113] Therefore, tumorigenesis induced by AOM-DSS treatment in MyD88$^{-/-}$ mice may differ from that in TLR2- or TLR4-deficient mice.

Besides TLRs, IL-18R, another upstream of MyD88 signaling, appears to be an important regulator of inflammation-associated tumorigenesis in the AOM-DSS model.[109] Mice deficient in IL-18 and IL-18R but not in IL-1R exhibit similar susceptibility to tumor development as MyD88$^{-/-}$ mice in the AOM-DSS model.[109] Similar to the MyD88$^{-/-}$ mice, these mice that are deficient in IL-18 and IL-18R exhibit severe inflammation but do not have increased IEC proliferation in response to AOM-DSS, suggesting that IL-18-induced IL-18R activation is responsible for the protective role of MyD88 signaling during colitis-associated tumorigenesis in this model.

Higher incidence of intestinal tumors in the AOM-DSS model has been observed in NLRP3$^{-/-}$ mice and NLRP6$^{-/-}$ mice who are unable to produce mature forms of IL-18 and IL-1β.[61,76,85,114] Similar tumorigenic phenotype has been reported in NLRC4$^{-/-}$ (NLR family CARD domain-containing protein 4: an another cytosolic member of NLR family) mice and caspase-1$^{-/-}$ mice.[76,114] As caspase-1 activation is the final step of NLR signaling, NLR-dependent protection against colitis-associated tumorigenesis is mediated by their endproducts. It is likely that IL-18 signaling integrates the role of NLRs in the resistance to intestinal tumor development as IL-1R$^{-/-}$ mice have similar susceptibility to intestinal tumor in the AOM-DSS model compared with WT mice.[109] NLRP12$^{-/-}$ mice also show increased susceptibility to AOM-DSS-induced colonic tumor that is associated with greater mucosal productions of IL-6, TNF-α, MIP2 (macrophage-inflammatory protein 2), IL-1β, and COX-2 due to increased activation of nuclear factor-κB, extracellular signal–regulated kinase, and STAT3.[115,116]

The major defect in these NLR-deficient mice lies in the regulation of epithelial cell proliferation, as these mice demonstrate increased proliferation of IECs during AOM-DSS treatment.[61,76,85,114] Similar increase in IEC proliferation during intestinal tumorigenesis has been reported in caspase-1$^{-/-}$ and NOD1$^{-/-}$ mice.[72,114] Although precise mechanisms underlying increased IEC proliferation in NLR-deficient mice remain to be explored, NLRP6$^{-/-}$ mice have shown greater expression of the genes associated with Wnt-signaling pathway in tumor tissue than tumors in WT mice in the AOM-DSS model.[62] Interestingly, a recent report demonstrated an increased susceptibility of NOD2$^{-/-}$ mice to intestinal tumorigenesis in response to AOM-DSS, which could be transmissible to WT mice by co-housing.[117] As the increased severity of DSS colitis in NLRP6$^{-/-}$ mice is also transmissible via commensal bacteria, the unique compositions of commensal bacteria may be involved in tumor susceptibility in this

**Table 4 Therapeutic challenges of PRR manipulation for murine colitis models**

| PRRs | Agonist/antagonist | Model of IBD used | Major effect |
|---|---|---|---|
| TLR2 | Agonist (Pam3CSK4)[52,54] | Acute DSS colitis, chronic MDR1α$^{-/-}$ | Prevention and treatment. Strengthen epithelial barrier. Increase TFF3 |
| TLR3 | Agonist (poly(I:C))[141] | Acute DSS colitis | Prevention. Involvement of Type I IFN? |
| TLR4 | Antagonist (1A6)[121]<br>Antagonist (CRX-526)[120] | Acute DSS colitis, chronic MDR1α$^{-/-}$<br>T-cell transfer colitis | Prevention. Blocking acute inflammatory infiltrate.<br>Blocking cytokine responses |
| TLR5 | Agonist (flagellin)[142] | Acute DSS colitis | Prevention if it is administered intraperitoneally |
| TLR9 | Agonist (CpG-ODN)[143,144]<br>Antagonist (AV-ODN)[60] | Acute DSS colitis, TNBS colitis, chronic DSS colitis, IL-10$^{-/-}$, T-cell transfer colitis | Prevention. Anti-apoptotic effect. Immuno-modulatory effect.<br>Induction of tolerance. Blocking host response to luminal bacterial CpG |
| NOD2 | Agonist (MDP)[123] | Acute DSS colitis, TNBS colitis | Downregulation of multiple TLR responses |

AV-ODN, adenoviral oligodeoxynucleotides; CpG-ODN, cytosine-phosphate-guanosine oligodeoxynucleotides; DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; IFN, interferon; IL, interleukin; MDP, muramyl dipeptide; MDR1, multidrug resistance; poly(I:C), polyinosinic-polycytidylic acid; PRR, pattern-recognition receptor; TFF3, trefoil factor; TLR, Toll-like receptor; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

model. Bone marrow chimera experiments have demonstrated important contributions of NLRP3 and NLRP6 in myeloid cells and NLRP12 in non-hematopoietic component to the protection against colitis-associated tumorigenesis.[61,76,115,116] Furthermore, an abnormal mucosal microenvironment has been proposed as a mechanism underlying increased tumorigenesis in these mice, because most NLR-deicient mice demonstrate more severe inflammation in response to DSS with greater expressions of tumorigenic cytokines (TNF-α, IL-6) and chemokines (keratinocyte chemoattractant, eotaxin, granulocyte colony-stimulating factor and monocyte chemotactic protein) in the intestinal mucosa compared with NLR-sufficient mice, while the lack of IL-18 production leads to a defective activation of anti-tumor signaling through IFN-γ and STAT1.[61,82] As IL-18 is known to facilitate anti-tumor immunity through induction of T helper type 1 and cytotoxic T-cell responses, IL-18-mediated suppression of intestinal tumorigenesis may also be involved in the regulation of anti-tumor immunity.[118] These results indicate that NLR signaling in the gastrointestinal tract promote multiple immune and non-immune programs that regulate neoplastic development in the setting of chronic inflammation.

## MANIPULATION OF PRR AS NOVEL THERAPY FOR COLITIS AND COLITIS-ASSOCIATED CANCER

Several TLR agonists and antagonists have been applied for the prevention and/or treatment of murine models of colitis (**Table 4**). In general, PRR signaling contributes to induction of regional inflammation but may promote cytoprotective and repair responses during mucosal injury in the gut. In addition, a portion of TLR4 signaling and most nucleotide-sensing TLRs may contribute to immuno-modulation through induction of type I IFNs. Therefore, selection of signaling targets and the timing of intervention are important to establish successful therapeutics for colitis.

Oral administration of a TLR2 agonist Pam3CSK4 has shown its therapeutic potential in DSS-induced colitis.[52] The protective effect of Pam3CSK4 is mediated through preservation of tight junctional epithelial barrier.[52,54,119] TLR2 stimulation also increases the colonic production of trefoil factor that facilitates wound healing and blocks apoptotic signaling.[54] Increased susceptibility of TLR2$^{-/-}$ mice to colitis-associated tumor further indicates a possible beneficial effect of Pam3CSK4 for cancer prevention during chronic colitis. Although TLR2 agonists have not yet entered clinical trials for human diseases, these results suggest that TLR2 signaling may be an ideal target for management strategy of IBD.

TLR4 antagonists (CRX-526; a synthetic lipid A mimetic molecule, 1A6; a specific monoclonal antibody) have been applied to prevent murine colitis.[120,121] Although TLR4 antagonist (1A6) suppressed induction of acute inflammatory infiltrate by blocking the expression of chemokines in the intestine when administered before colitis, it delayed mucosal healing when administered to established colitis.[121] The TLR4 blocking strategy did not ameliorate the chronic model of T-cell transfer colitis, but administration of 1A6 during recovery phase of colitis significantly prevented tumor development in the AOM-DSS model.[28,121] Therefore, combination therapies with cytoprotective agents may be required for TLR4 blocking strategy to avoid the delay of mucosal healing. TLR4 antagonists are currently evaluated in clinical trials for sepsis cases.[122]

*In vivo* manipulation of NLRs have also been examined. NOD2 stimulation by systemic administration of muramyl dipeptide appears to be beneficial in mouse models of colitis.[123] Glyburide (glibenclamide), a sulfonylurea drug for the treatment of type 2 diabetes, is known to inhibit ATP-sensitive $K^+$ channels and thus can prevent NLRP3 inflammasome activation.[124] Glyburide has been shown to prevent ventilator-induced lung injury in mice and delay endotoxin-induced lethality in mice, but it has not been applied for animal models of colitis.[124,125]

## CONCLUSION

PRR signaling has significant roles in intestinal homeostasis, which is mainly composed of the maintenance of commensals. Extensive studies in animal models of colitis have provided several key points of the contribution of individual PRRs and their effector pathways in the pathogenesis of colitis. Epithelial PRR signaling mainly promotes mucosal protection through induction of pathways that leads to cell proliferation and survival or cytoprotection in response to mucosal injury. By contrast, it is likely that PRR signaling in hematopoietic cells is responsible for induction of local inflammation in response to invading pathogens. These PRR signals may be enhanced during chronic inflammation in the intestine. Continuous activation of abnormal PRR signaling leads to the development of neoplasms. In this regard, PRR signaling is involved in multiple aspects of intestinal tumorigenesis as well as host anti-tumor immunity. Manipulation of PRR signaling as therapeutic strategies of human diseases has just begun. Not only targeting particular PRR signaling but targeting upstream signaling or downstream effector molecules such as caspase-1, IL-1β or IL-18 may be beneficial to develop novel strategies for managing IBD and prevention of colorectal cancer in those patients.

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### DISCLOSURE

The authors declared no conflict of interest.



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