Original Article

Crosstalk between NOD2 and TLR2 suppresses the development of TLR2-mediated experimental colitis

Natsuki Okai,[1] Yasuhiro Masuta,[1] Yasuo Otsuka,[1] Akane Hara,[1] Sho Masaki,[1] Ken Kamata,[1] Kosuke Minaga,[1] Hajime Honjo,[1] Masatoshi Kudo,[1] and Tomohiro Watanabe[1,2,*]

[1]Department of Gastroenterology and Hepatology, Kindai University Faculty of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan
[2]Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan

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Nucleotide-binding oligomerization domain 2 (NOD2) is an intracellular sensor for muramyl dipeptide (MDP), a degradation product of bacterial cell wall peptidoglycan (PGN). PGN stimulates cell-surface Toll-like receptor 2 (TLR2) independently of NOD2, indicating the presence of crosstalk between extracellular TLR2 and intracellular NOD2 upon exposure to PGN. NOD2-deficient mice were sensitive, while TLR2-deficient mice were resistant to experimental colitis induced by intrarectal administration of PGN. Severe colitis in NOD2-deficient mice was accompanied by increased expression of nuclear factor-kappa B-dependent cytokines and decreased expression of autophagy-related 16-like 1 (ATG16L1). MDP activation of NOD2 enhanced autophagy mediated by TLR2 in human dendritic cells. mRNA expression of TLR2 tended to be higher in the colonic mucosa of patients with active ulcerative colitis compared to that of those in remission. Induction of remission was associated with increased mRNA expression of ATG16L1 in both ulcerative colitis and Crohn's disease patients. Conversely, mRNA expression of receptor-interacting serine/threonine-protein kinase 2 was higher in the inflammatory colonic mucosa of patients with active disease than in the non-inflamed mucosa of patients in remission, in both ulcerative colitis and Crohn's disease. These findings highlight the role of NOD2-TLR2 crosstalk in the immunopathogenesis of colitis.

***Key Words*: NOD2, TLR2, ATG16L1, colitis**

Peptidoglycan (PGN) is a major component of the bacterial cell wall in both gram-negative and gram-positive bacteria.[1–3] Bacterial cell wall PGN is a well-established Toll-like receptor 2 (TLR2) ligand as shown by the fact that mice deficient in TLR2 exhibit defective proinflammatory cytokine responses against PGN.[4] Intracellular pattern-recognition receptors (PRRs) sense degradation products derived from PGN, independently of TLR2. The degradation of PGN *in vivo* or *in vitro* results in the formation of muropeptides with the ability to stimulate intracellular PRRs, including nucleotide-binding oligomerization domain (NOD)-like receptors.[1–3] One of these prototypical muropeptides, muramyl dipeptide (MDP), is recognized by intracellular NOD2, one of the NOD-like receptors.[1–3,5] Thus, there is a dual PRR recognition system operating when exposed to PGN. PGN is recognized by cell-surface TLR2, whereas intracellular NOD2 is activated simultaneously by sensing MDP.

NOD2 plays an essential role in maintaining tolerogenic responses against gut bacteria and is primarily expressed in dendritic cells (DCs) and macrophages.[1,6] Loss-of-function mutations in NOD2 are the strongest risk factors for Crohn's disease (CD), a chronic inflammatory disorder driven by excessive proinflammatory cytokine responses against gut bacteria.[1,6] The molecular mechanism accounting for NOD2-mediated tolerance inhibiting the development of CD is mediated by the prior activation of NOD2 by MDP in DCs and macrophages, which markedly reduces the production of proinflammatory T helper type 1 (Th1) cytokine responses, including TNF-α, IL-12, and IL-23 on subsequent stimulation with TLR2, TLR3, TLR4, TLR5, and TLR9 ligands.[5,7–14] Activation of NOD2 efficiently induces the expression of interferon regulatory factor 4 (IRF4) and thereby suppresses the production of nuclear factor-kappa B (NF-κB)-dependent Th1 cytokines in DCs. NOD2 functions as a negative regulator of multiple TLRs-mediated inflammatory responses, and the presence of CD-associated NOD2 mutations or NOD2 deficiency leads to colitis due to excessive proinflammatory cytokine responses triggered by TLRs.[11,12] Collectively, the crosstalk between NOD2 and multiple TLRs induced by exposure to gut bacteria is necessary to maintain intestinal immune homeostasis.

NOD2 mediates tolerogenic responses against multiple TLRs when DCs are pre-incubated with MDP before stimulation with TLR ligands. However, TLR2-mediated cytokine responses are suppressed if cells are pre-stimulated with MDP followed by subsequent exposure to TLR2 ligands or if they are simultaneously stimulated with MDP and TLR2 ligands.[5,10,15,16] Thus, crosstalk between NOD2 and TLR2 differs from that between NOD2 and other TLRs. In this study, we explored the role played by crosstalk between NOD2 and TLR2 in experimental murine colitis and human inflammatory bowel disease (IBD).

Materials and Methods

PGN colitis. Female C57BL/6 mice, aged 6–8 weeks, were obtained from Japan SLC, Inc. (Hamamatsu, Japan). NOD2-deficient ($NOD2^{-/-}$) or TLR2-deficient ($TLR2^{-/-}$) mice, as previously described,[9,17,18] were utilized. The mice were reared under specific-pathogen-free conditions, and all animal experiments were approved by the Review Board of Kyoto University Graduate School of Medicine. To induce PGN colitis, mice were first pretreated with intrarectal administration of 50% ethanol (100 μl) and intrarectally challenged with 1 mg of PGN (InvivoGen, San Diego, CA) 8 h after the ethanol treatment, following established protocols.[7] Bone marrow (BM)-chimeric mice were generated by transplanting BM cells from $NOD2^{-/-}$ mice into green fluorescence protein transgenic (GFP-Tg) mice

*To whom correspondence should be addressed.
E-mail: tomohiro@med.kindai.ac.jp

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or by transplanting BM cells from GFP-Tg mice into NOD2$^{-/-}$ mice, as previously described.[8,17] These BM-chimeric mice were challenged with PGN colitis two months after the BM transplantation.

Pathological and immunohistochemical analyses. The colon tissues were harvested three days after inducing PGN colitis and then fixed with 10% formalin. After deparaffinization, the colonic sections were stained with hematoxylin and eosin (H&E). Pathological scoring for PGN colitis was carried out following the same method used for trinitrobenzene sulfonic acid (TNBS) colitis.[9,11] The expression of autophagy-related 16-like 1 (ATG16L1) and phospho-IκBα (p-IκBα) was visualized using the DAKO EnVision+ System (DAKO Japan, Tokyo, Japan) with mouse anti-ATG16L1 antibody (Ab; MBL, Tokyo, Japan) and mouse anti-p-IκBα Ab (CST, Cambridge, MA), as previously described.[8]

Isolation of colonic lamina propria mononuclear cells. Colonic lamina propria mononuclear cells (cLPMNCs) were isolated in accordance with an established protocol.[7,9,11] cLPMNCs (2×10^6/ml) were stimulated with *Staphylococcus aureus* (SAC, 1:10,000; Calbiochem, La Jolla, CA) and IFN-γ (50 ng/ml; BD Biosciences, San Jose, CA) to induce IL-12/23 p40 production.[7] cLPMNCs were also cultured with anti-CD3 monoclonal Ab (5 μg/ml; BD Biosciences) for IFN-γ production.[9,18] Cells were cultured in complete RPMI medium for 60 h. Cytokine levels in the culture supernatants were measured using enzyme-linked immunosorbent assay (ELISA).

Human monocyte-derived DCs. Human monocyte-derived DCs were prepared using peripheral blood monocytes from three healthy volunteers, as previously described.[19] This study was approved by the Ethical Review Board of Kyoto University Graduate School of Medicine. DCs were stimulated with PAM_3CSK4 (PAM, 10 μg/ml; InvivoGen) and/or MDP (50 μg/ml; InvivoGen) in the presence or absence of wortmannin (2.5 μM; InvivoGen) or dimethyl sulfoxide (DMSO) in complete RPMI medium for 24 h. Culture supernatants were subjected to ELISA for measurement of IL-12/23p40 and IL-6.

ELISA. The cell supernatants were subjected to ELISA using a murine IFN-γ ELISA kit (eBioscience, San Diego, CA), a murine IL-12/23p40 ELISA kit (eBioscience), a human IL-12/23p40 ELISA kit (BD Biosciences), and a human IL-6 ELISA kit (BD Biosciences), as previously described.[8,18,20]

Immunoblotting. Immunoblotting was performed as previously described.[11,18] Protein extracts were prepared from cLPMNCs (2×10^6 cells) and human DCs (1×10^6 cells).[11,18] Protein extracts (10 μg) were applied to NuPAGE 4–12% Bis-Tris Mini Protein Gels (Invitrogen, Carlsbad, CA), followed by transfer to a nitrocellulose membrane (Invitrogen). Mouse anti-p-IκBα Ab (CST), rabbit anti-LC3 Ab (MBL), and goat anti-actin Ab (Santa Cruz Biotechnology, Dallas, TX) were used as primary Abs.

Autophagy monitoring. Human DCs (5×10^5/ml) were incubated with BacMam LC3B-RFP baculovirus reagent (Premo Autophagy Sensor Kit; Invitrogen) and PAM (10 μg/ml) and/or MDP (50 μg/ml) for 16 h, as previously described.[21] Formation of LC3 puncta visualized by red fluorescence was assessed by Keyence fluorescence microscopy (Bio-zero, BZ-8100; Keyence, Osaka, Japan).

Patients and quantitative reverse transcription polymerase chain reaction (qRT-PCR). Patients with active ulcerative colitis (UC) ($n = 20$) and UC in remission ($n = 20$), and patients with active CD ($n = 10$) and CD in remission ($n = 10$) were recruited for this study.[18] The patient characteristics have been reported previously.[18] The study was approved by the Review Board of Kindai University Faculty of Medicine. Written informed consent was obtained from each patient. mRNA was isolated from colonic biopsy samples obtained during endoscopy using TRIzol reagent (Invitrogen), as previously described.[9,18] qRT-PCR was performed after reverse transcription of mRNA into cDNA using Superscript III (Invitrogen). SYBR Green-based qPCR was performed using a LightCycler 480 system (Roche, Tokyo, Japan).[9,18,22] Target primers for ATG16L1, receptor-interacting serine/threonine-protein kinase 2 (RIPK2) and TLR2 were purchased from Qiagen (Hilden, Germany). *ACTB* mRNA expression was used as the internal control.[9,18,22]

Statistical analyses. Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA).[23] The Mann–Whitney *U* test, a nonparametric version of the independent samples *t* test, was used to evaluate the differences between groups. The Kruskal–Wallis test, a nonparametric version of one-way analysis of variance, was used to evaluate the significance of differences between multiple comparisons. For post hoc analysis, the Bonferroni-corrected Mann–Whitney *U* test was performed for comparison between groups. P values <0.05 were considered statistically significant.

Results

NOD2-deficient mice are sensitive to PGN colitis. NOD2 is a negative regulator of TLR2-mediated Th1 responses.[5,10] MDP, a NOD2 ligand, is a degradation product of PGN, a TLR2 ligand; thus PGN administration can stimulate both cell-surface TLR2 and intracellular NOD2.[2] Intrarectal administration of 50% ethanol followed by administration of soluble PGN per rectum causes colitis (PGN colitis).[7] In initial studies, we explored the effects of crosstalk between NOD2 and TLR2 on PGN colitis using NOD2$^{-/-}$ mice and TLR2$^{-/-}$ mice.

As depicted in Fig. 1A, there was minimal body weight loss observed in TLR2$^{-/-}$ mice challenged with PGN colitis, implying that the development of PGN colitis necessitates the activation of TLR2. Upon exposure to PGN colitis, NOD2$^{-/-}$ mice exhibited significant body weight loss in comparison to the control C57BL/6 mice and the TLR2$^{-/-}$ mice. Moreover, we observed the destruction of crypt architecture accompanied by immune cell infiltration in both NOD2$^{-/-}$ mice and C57BL/6 mice. However, the degree of colon injury was notably higher in NOD2$^{-/-}$ mice than in C57BL/6 mice, as assessed using a semi-quantitative colitis scoring system (Fig. 1B and C). In contrast, TLR2$^{-/-}$ mice displayed a high level of resistance to PGN colitis in pathological analyses. These findings collectively suggest that the activation of NOD2 through the detection of MDP, a degradation product of PGN, serves to inhibit the development of PGN colitis, which is initiated by the activation of TLR2.

NOD2 is expressed in both hematopoietic cells and epithelial cells.[24] To determine whether NOD2 expression in hematopoietic cells and/or non-hematopoietic cells plays a pathogenic role in the development of PGN colitis, we created two types of BM-chimeric mice: irradiated NOD2-intact GFP-Tg mice transplanted with NOD2$^{-/-}$ BM cells (referred to as NOD2$^{-/-}$>GFP-Tg mice) and irradiated NOD2$^{-/-}$ mice transplanted with NOD2-intact GFP-Tg BM cells (referred to as GFP-Tg>NOD2$^{-/-}$ mice). The body weight curves of these two types of BM-chimeric mice were similar, as shown in Fig. 1D. Consistent with the body weight curves, there were no significant differences in pathology scores between the two types of BM-chimeric mice (data not shown).

Enhanced sensitivity to PGN colitis is associated with reduced expression of ATG16L1. Having confirmed that NOD2 deficiency leads to the development of severe PGN colitis, we proceeded to investigate the involvement of NF-κB-dependent Th1 responses in both NOD2$^{-/-}$ mice and TLR2$^{-/-}$ mice. We observed significantly higher expression of p-IκBα in cLPMNCs from NOD2$^{-/-}$ mice compared to C57BL/6 mice and TLR2$^{-/-}$ mice (Fig. 2A). Furthermore, in Fig. 2C, we noted an increased number of p-IκBα^{+} epithelial cells in the colons of NOD2$^{-/-}$ mice in comparison to those of TLR2$^{-/-}$ mice and C57BL/6 mice. This increased activation of NF-κB appeared to

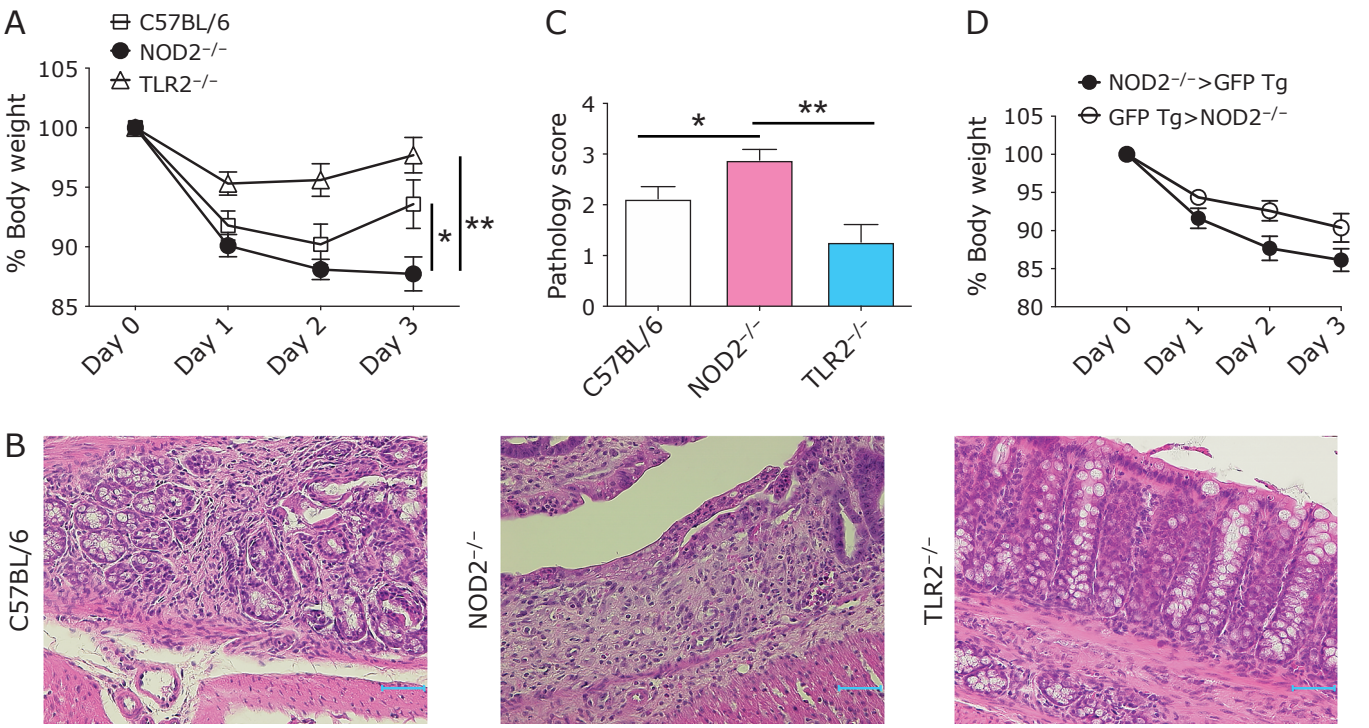

Fig. 1. Mice deficient in nucleotide-binding oligomerization domain 2 develop severe peptidoglycan colitis. C57BL/6 mice (n = 15), NOD2-deficient mice (NOD2$^{-/-}$, n = 17), and Toll-like receptor 2-deficient mice (TLR2$^{-/-}$, n = 14) were challenged with peptidoglycan (PGN) colitis. (A) Body weight curve. (B) Hematoxylin and eosin staining of colonic tissues obtained on day 3. Scale bar; 50 μm. (C) Pathology score for PGN colitis. (D) Two types of bone marrow chimeric mice were created. Irradiated green fluorescence protein transgenic (GFP-Tg) mice transplanted with bone marrow cells from NOD2$^{-/-}$ mice (NOD2$^{-/-}$>GFP-Tg, n = 7) and irradiated NOD2$^{-/-}$ mice transplanted with bone marrow cells from GFP-Tg mice (GFP-Tg>NOD2$^{-/-}$, n = 9) were challenged with PGN colitis 2 months after bone marrow transplantation. Body weight curves are shown. Results are expressed as mean ± SEM, *p<0.05, **p<0.01.

coincide with proinflammatory Th1 responses in NOD2$^{-/-}$ mice. When cLPMNCs isolated from NOD2$^{-/-}$ mice challenged with PGN colitis were subjected to *in vitro* stimulation, they produced substantial amounts of Th1 cytokines, including IL-12/23p40 and IFN-γ (Fig. 2B). In light of these findings, it is likely that NF-κB-dependent Th1 responses play a pivotal role in the pathogenesis of severe PGN colitis in NOD2$^{-/-}$ mice.

Activation of NOD2 induces autophagy through interaction with ATG16L1.[25,26] We previously reported that the molecular interaction between NOD2 and ATG16L1 negatively regulates TLR2-mediated NF-κB responses.[27] Importantly, on challenge with PGN colitis, the accumulated immune cells were negative for ATG16L1 staining in the colonic mucosa of NOD2$^{-/-}$ mice (Fig. 2C). In contrast, immune cells were positive for ATG16L1 staining in the colonic mucosa of TLR2$^{-/-}$ and C57BL/6 mice. Collectively, these findings suggest that enhanced sensitivity of PGN colitis in NOD2$^{-/-}$ mice is associated with upregulation of NF-κB-dependent Th1 cytokine responses and downregulation of ATG16L1 expression.

Co-stimulation with NOD2 and TLR2 enhances autophagy. Activation of ATG16L1 plays a critical role in autophagic responses.[25,26] Severe PGN colitis was accompanied by reduced expression of ATG16L1 in NOD2$^{-/-}$ mice. In contrast, enhanced ATG16L1 expression was seen in C57BL/6 mice resistant to PGN colitis. Given the presence of crosstalk between NOD2 and TLR2 in C57BL/6 mice, but not NOD2$^{-/-}$ mice, we assessed whether co-stimulation with NOD2 and TLR2 enhances autophagic responses through activation of ATG16L1. To this end, we used an established NOD2 and TLR2 co-stimulation system, in which human monocyte-derived DCs were cultured with MDP and PAM, a pure TLR2 ligand without NOD2-stimulating capacity.[7,10]

As shown in Fig. 3A, immunoblotting studies revealed a higher ratio of LC3-II/LC3-I expression and LC3-II expression in human DCs treated with both MDP and PAM than in those treated with MDP or PAM alone. In addition, formation of LC3 puncta was enhanced in human DCs treated with both MDP and PAM compared with DCs treated with MDP or PAM alone, as assessed by fluorescence microscopy (Fig. 3B). These findings suggest that autophagic responses are enhanced in human DCs co-treated with NOD2 and TLR2 ligands compared with DCs treated with NOD2 or TLR2 ligands alone. Thus, these studies using human DCs co-stimulated with NOD2 and pure TLR2 ligands support the hypothesis that NOD2-TLR2 crosstalk activated by PGN promotes autophagic responses.

Inhibition of autophagy by wortmannin enhances proinflammatory cytokines. Wortmannin is a potent inhibitor of phosphatidylinositol 3-kinase (PI3K). PI3K is essential for induction of autophagy.[28,29] We examined whether inhibition of autophagy increases production of proinflammatory cytokines mediated by TLR2. To this end, human DCs were stimulated with PAM or MDP in the presence of wortmannin or DMSO. Compared with DMSO, inhibition of PI3K by wortmannin increased production of IL-12/23p40 and IL-6 on stimulation with PAM, although the difference was not statistically significant due to the highly variable cytokine responses in each subject (Fig. 4A). Thus, inhibition of autophagy by wortmannin promotes TLR2-mediated Th1 responses. Taken together, these experiments using PGN colitis and human DCs suggest that the NOD2-TLR2 crosstalk activated by sensing of PGN negatively regulates Th1 responses to bacterial cell walls partially through expression of ATG16L1 and induction of autophagy, and that NOD2 deficiency leads to the development of colitis through enhanced Th1 responses to TLR2 ligands.

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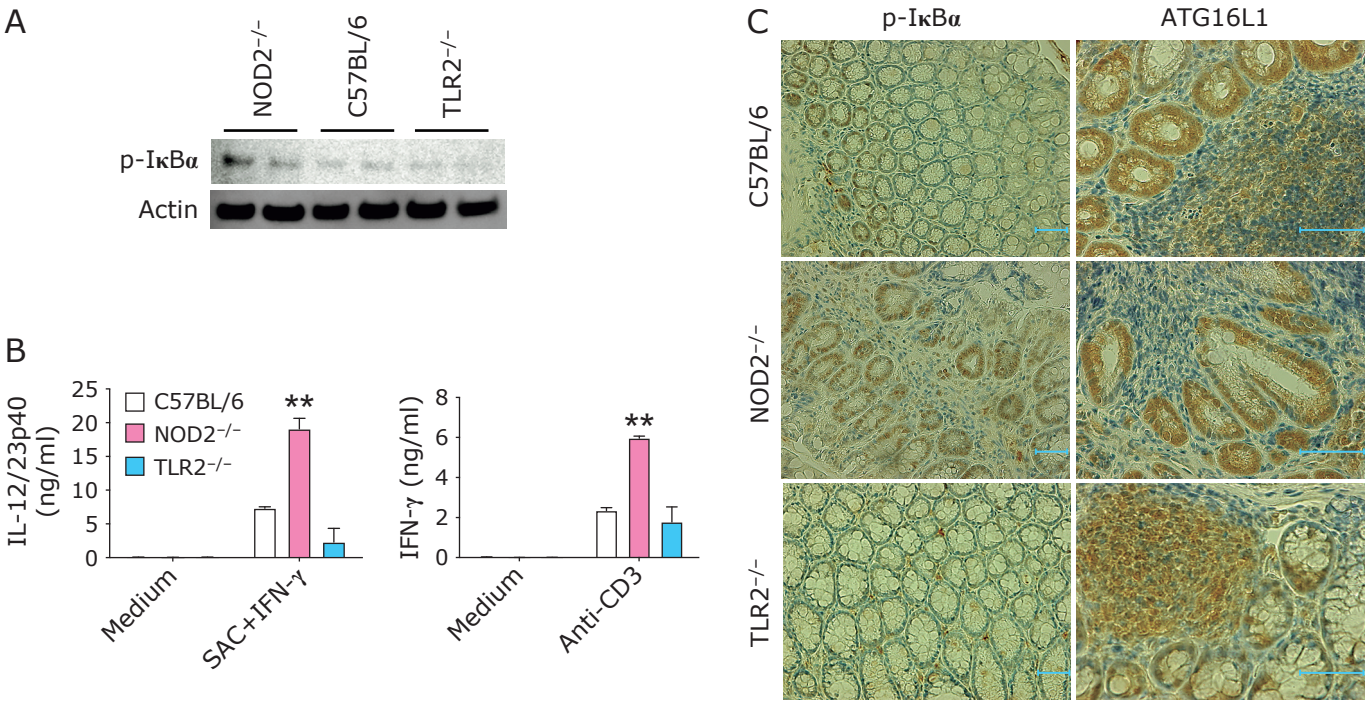

Fig. 2. Severe peptidoglycan colitis in mice deficient in nucleotide-binding oligomerization domain 2 is associated with enhanced activation of nuclear factor-κB and reduced expression of autophagy-related 16-like 1. Peptidoglycan colitis was induced as described in Fig. 1. (A) Protein expression of phospho-IκBα (p-IκBα) and actin in colonic lamina propria mononuclear cells (cLPMNCs). Each lane corresponds to the expression of p-IκBα and actin in each mouse. (B) cLPMNCs (2×10^6/ml) were stimulated with *Staphylococcus aureus* (SAC, 1/10,000) and IFN-γ (50 ng/ml) or anti-CD3 monoclonal antibody (5 μg/ml) for 60 h. Culture supernatants were subjected to enzyme-linked immunosorbent assays for measurement of IL-12/23p40 and IFN-γ. Results shown are representative of two independent experiments. (C) Colonic expression of p-IκBα and autophagy-related 16-like 1 (ATG16L1). Scale bar; 50 μm. Results shown are representative of three independent experiments. Results are expressed as the mean ± SEM, $^{**}p<0.01$.

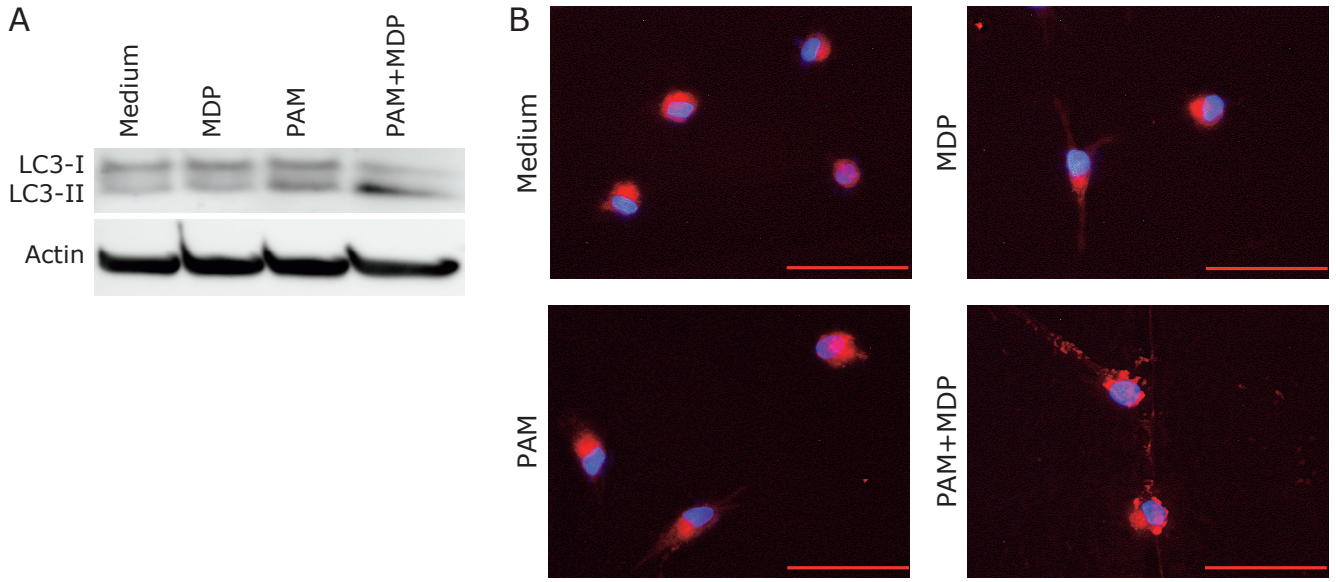

Fig. 3. Co-stimulation with nucleotide-binding oligomerization domain 2 and Toll-like receptor 2 induces autophagy. Human monocyte-derived dendritic cells (DCs, 1×10^6/ml or 5×10^5/ml) were stimulated with muramyl dipeptide (MDP, 50 μg/ml) and/or PAM_3CSK4 (PAM, 10 μg/ml) for 16 h. (A) Protein expression of LC3-I/II and actin. (B) Formation of autophagosome shown as red fluorescence. LC3 puncta formation is enhanced in DCs treated with both PAM and MDP. Scale bar; 50 μm. Results shown are representative of two independent experiments.

ATG16L1 mRNA expression is enhanced in patients with UC or CD in remission. Finally, we addressed the clinical relevance of these findings in patients with UC and CD, including patients with active disease and disease in remission. To this end, we used colonic biopsy samples from patients with CD and UC, as previously described.[18] mRNA expression of proinflammatory cytokines was higher in patients with active IBD than in patients in remission, as shown in our previous study.[18] As

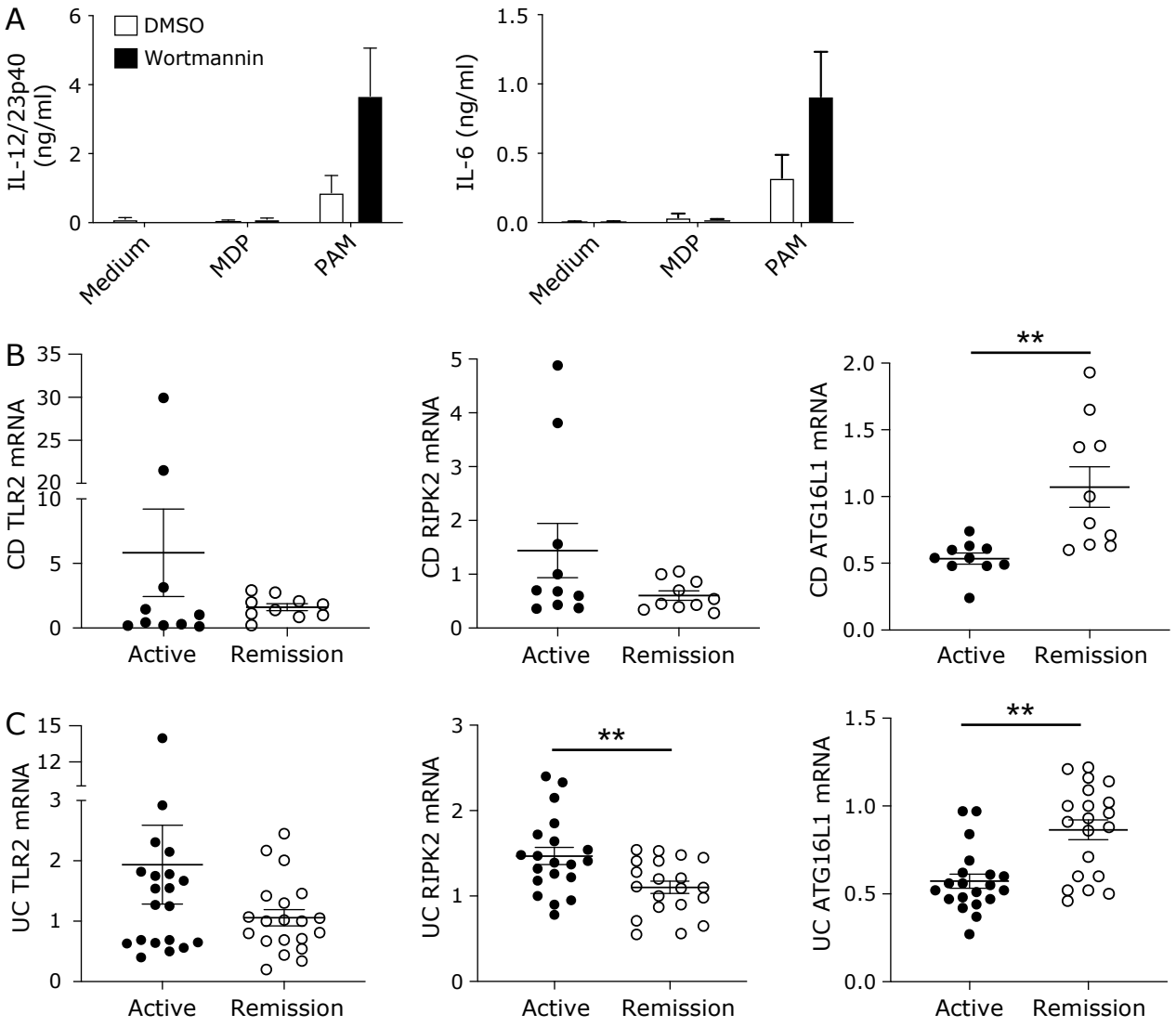

Fig. 4. mRNA expression of autophagy-related 16-like 1 is enhanced in the colonic mucosa of patients with ulcerative colitis and Crohn's disease in remission. (A) Human monocyte-derived dendritic cells (DCs, 1 × 10^6/ml) were stimulated with muramyl dipeptide (MDP, 50 μg/ml) or PAM_3CSK4 (PAM, 10 μg/ml) in the presence of wortmannin (2.5 μM) for 24 h. Monocytes were isolated from healthy volunteers (n = 3). Culture supernatants were subjected to enzyme-linked immunosorbent assays to measure IL-12/23p40 and IL-6. (B, C) mRNA expression of Toll-like receptor 2 (TLR2), receptor-interacting serine/threonine-protein kinase 2 (RIPK2), and autophagy-related 16-like 1 (ATG16L1) in patients with Crohn's disease (CD, active phase; n = 10, remission phase; n = 10) and ulcerative colitis (UC, active phase; n = 20, remission phase; n = 20). Each dot corresponds to the value of each patient. Results are expressed as the mean ± SEM, $^{**}p<0.01$.

shown in Fig. 4B and C, mRNA expression of TLR2 was comparable between patients with active CD and those with CD in remission, whereas TLR2 mRNA expression tended to be higher in patients with active UC than in those with UC in remission. Similar data were observed for mRNA expression of TLR4 (data not shown) and no significant difference was seen in mRNA expression of NOD2 between patients with active IBD and those with IBD in remission, as shown in our previous study.[18]

RIPK2 is a downstream signaling molecule for NOD2, TLR2, and TLR4.[9,12,27,30] Consistent with our previous study, mRNA expression of RIPK2 was higher in patients with active CD and UC than in patients in remission, although in patients with CD the difference between those with active disease and those in remission was not statistically significant due to the limited number of patients.[9] In contrast, in both CD and UC mRNA expression of ATG16L1 was significantly higher in patients with active disease than in patients with disease in remission (Fig. 4B and C). These data using human IBD samples show that induction of remission was accompanied by increased and decreased mRNA expression of ATG16L1 and RIPK2, respectively.

Discussion

NOD2 and TLRs are major PRRs for the detection of components of gut bacteria.[1,2,6,24] Our research, as well as studies by others, has demonstrated that chronic stimulation of NOD2 by MDP mediates tolerance to multiple TLRs and bacterial products.[5,7–14] This NOD2-induced tolerance contributes to the generation of a suppressive immune environment in the gut. Notably, CD-associated NOD2 mutations cause chronic inflammation due to excessive proinflammatory cytokine responses on exposure to bacterial components and TLR ligands. Therefore, the crosstalk between NOD2 and TLRs underlies the maintenance of immune homeostasis against gut bacteria. Negative regulation of NOD2 on TLRs operates when activation of NOD2 occurs prior to that of TLRs.

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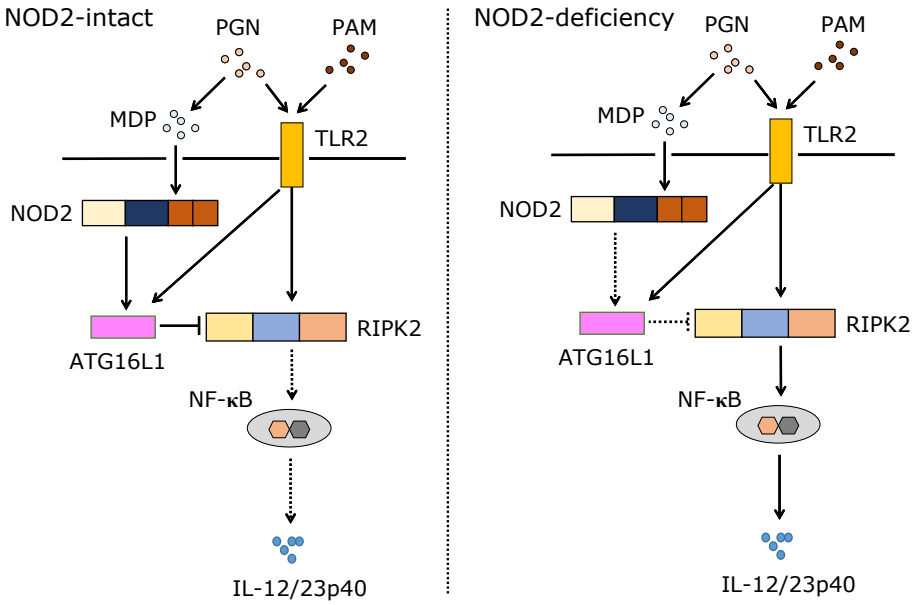

Fig. 5. Mechanisms of crosstalk between nucleotide-binding oligomerization domain 2 and Toll-like receptor 2 (TLR2). Muramyl dipeptide (MDP) is a degradation product of bacterial cell wall peptidoglycan (PGN). Activation of cell-surface TLR2 by PGN and intracellular NOD2 by MDP is simultaneously induced on exposure to PGN in dendritic cells and macrophages. Sensing of MDP by NOD2 negatively regulates nuclear factor-kappa B (NF-κB)-dependent production of IL-12/23p40 induced by sensing of PGN or PAM_3CSK4 (PAM) by TLR2. Autophagy-related 16-like 1 (ATG16L1) is involved in the crosstalk between NOD2 and TLR2. In the case of NOD2-deficiency, lack of the crosstalk between NOD2 and TLR2 enhances NF-κB-dependent production of IL-12/23p40 mediated by TLR2. Left; NOD2-intact cells, right; NOD2-deficient cells. RIPK2; receptor-interacting serine/threonine-protein kinase 2.

The NOD2-TLR2 crosstalk is unique because PGN, a component of bacterial cell walls, can activate both cell-surface TLR2 and intracellular NOD2. NOD2 is activated by sensing of a degradation product of PGN (MDP, Fig. 5). In this study, we clarified the role of NOD2-TLR2 crosstalk in the development of PGN colitis and human IBD. Here we provide evidence that the NOD2-TLR2 crosstalk activated on induction of PGN colitis inhibits colonic inflammatory responses in NOD2-intact mice (Fig. 5A). In contrast, $NOD2^{-/-}$ mice developed severe PGN colitis due to the lack of the NOD2-TLR2 crosstalk. This was accompanied by upregulation of expression of NF-κB-dependent Th1 cytokines and downregulation of expression of ATG16L1 (Fig. 5B). Consistent with the results of the PGN colitis experiment, ATG16L1 mRNA expression is significantly lower in the colonic mucosa of patients with active IBD than in that of patients in remission. Thus, our data support the involvement of NOD2-TLR2 crosstalk in the maintenance of immune homeostasis to gut bacterial products. As with PGN colitis, defective crosstalk between NOD2 and TLR2 has been shown to result in the development of PGN uveitis due to excessive Th1 responses.[31]

In our previous study, we demonstrated that NOD2-Tg mice, under the control of the MHC class II promoter, were resistant to PGN colitis compared to their wild littermate control mice.[7] Furthermore, cLPMNCs isolated from NOD2-Tg mice displayed reduced production of IL-12 compared to control mice.[7] The results of the present study using $NOD2^{-/-}$ mice align with those of the previous study involving NOD2-Tg mice. Additionally, we confirmed that the development of PGN colitis hinges on the activation of TLR2. Therefore, NOD2-TLR2 crosstalk efficiently suppresses TLR2-mediated colitis.

An important question raised by the present study is whether the negative regulation of NOD2 on TLR2-mediated inflammatory responses alone is sufficient to prevent colitis driven by gut bacteria. Given that bacterial components activate a wide range of TLRs, leading to the induction of colitogenic cytokine responses, it becomes evident that the prevention of colitis necessitates NOD2-mediated negative regulation of multiple TLRs, extending beyond TLR2. In our prior research, we established an experimental colitis model induced by the adoptive transfer of ovalbumin (OVA)-specific $CD4^+$ T cells and colonization with *Escherichia coli* expressing OVA.[10] In this bacterial antigen-specific colitis model, we observed that NOD2-deficient mice developed severe colitis characterized by heightened Th1 responses.[10] Importantly, the development of severe bacterial antigen-induced colitis was effectively suppressed in mice lacking both NOD2 and TLR2.[10] This suggests that certain forms of experimental colitis may originate from an inadequate crosstalk between NOD2 and TLR2. Consistent with this hypothesis, it is worth noting that NOD2-Tg mice have been reported to exhibit resistance not only to PGN colitis but also to TNBS colitis.[7]

NOD2 plays a pivotal role as a negative regulator of multiple TLRs, including TLR2, TLR3, TLR4, TLR5, and TLR9.[5,7–14] The induction of IRF4 expression triggered by NOD2 activation is of paramount importance in NOD2-mediated suppression of these TLRs.[11,12,32,33] IRF4 forms a binding partnership with RIPK2, a downstream signaling molecule of TLRs, effectively inhibiting polyubiquitination of RIPK2 and thereby dampening NF-κB-dependent cytokine responses.[11,12,32,33] The activation of NOD2 by MDP has been shown to inhibit the development of TNBS colitis, dextran sodium sulfate colitis, colorectal cancer, and obesity-induced insulin resistance in an IRF4-dependent manner.[11,12,32,33] While IRF4 induced by NOD2 activation efficiently suppresses NF-κB-dependent cytokine responses mediated by TLRs, our prior research has reported that transfection of ATG16L1 cDNA reduces NF-κB activation by TLR2, albeit not by TLR4, in reporter gene assays.[27,30] Mechanistically, ATG16L1 physically interacts with RIPK2 and collaborates synergistically with IRF4 to suppress TLR2-mediated NF-κB activation through the inhibition of RIPK2 polyubiquitination.[27,30] Consequently, it is apparent that TLR2-mediated inflam-

matory responses may exhibit high sensitivity to NOD2-mediated suppression not solely due to the influence of IRF4 but also because ATG16L1 functions as an effector molecule. In the present study, exacerbation of TLR2-dependent PGN colitis coincided with reduced expression of ATG16L1 in NOD2$^{-/-}$ mice, while the induction of remission in patients with IBD was correlated with the upregulation of ATG16L1 and the downregulation of RIPK2. These findings lend support to the hypothesis that NOD2-mediated suppression operates through the concerted action of both IRF4 and ATG16L1.

The *ATG16L1* T300A variant increases the risk of CD due to defective autophagic responses.[29] Our previous studies showed that suppression of RIPK2 polyubiquitination by ATG16L1 is involved in inhibition of TLR2-mediated inflammatory responses.[27,30] However, it is unknown whether ATG16L1 inhibits TLR2-mediated inflammatory responses through induction of autophagy. Given that expression of ATG16L1 was reduced in NOD2$^{-/-}$ mice on challenge with PGN colitis, alterations in autophagic responses might be associated with the development of severe PGN colitis in NOD2$^{-/-}$ mice. However, detection of autophagy *in vivo* is technically difficult and we did not detect autophagy in the colonic mucosa of mice with PGN colitis. To examine the effects of NOD2-TLR2 crosstalk on autophagy, we used an *in vitro* co-culture system in which human monocyte-derived DCs were co-stimulated with MDP (an NOD2 ligand) and PAM (a pure TLR2 ligand). The conversion of LC3-I to LC3-II was detected in DCs co-stimulated with MDP and PAM, but not in DCs stimulated with either MDP or PAM alone. In addition, formation of LC3 puncta was enhanced in DCs co-stimulated with MDP and PAM. These autophagy monitoring studies support the idea that the crosstalk between NOD2 and TLR2 enhances autophagic responses.[34] Together with the findings that inhibition of autophagy by wortmannin increased production of IL-12/23p40 and IL-6 by human DCs, these findings suggest that enhanced autophagy contributes to suppression of TLR2-mediated inflammatory responses mediated by NOD2-TLR2 crosstalk. Although autophagic responses induced by NOD2 depends on activation of ATG16L1,[25,26] it remains unknown whether the NOD2-TLR2 crosstalk induces autophagy in an ATG16L1-dependent manner.

We addressed the clinical relevance of the NOD2-TLR2 crosstalk using human IBD samples. We used colonic biopsy samples from patients with active IBD and patients with IBD in remission.[18] In our previous study, no significant differences in NOD2 mRNA expression were observed between patients with active IBD and patients with IBD in remission.[18] Colonic mucosa of patients with active UC showed higher mRNA expression of TLR2 than that in patients with UC in remission, whereas patients with active CD and those with CD in remission showed comparable levels of mRNA expression. Consistent with our previous report, mRNA expression of RIPK2, a downstream signaling molecule for TLR2 and TLR4, was directly proportional to disease activity in patients with IBD.[9] In contrast, mRNA expression of ATG16L1 was inversely correlated to disease activity in patients with IBD. Thus, these qRT-PCR studies using human IBD samples suggest possible involvement of reciprocal regulation of RIPK2 and ATG16L1 expression in the immunopathogenesis of human IBD. Notably, activation of RIPK2 and ATG16L1 is tightly regulated by sensing of bacterial components by TLRs and NOD2. Collectively, these findings highlight the role of NOD2-TLR2 crosstalk in the immunopathogenesis of colitis.

Author Contributions

Conceptualization: NO, TW; Methodology: NO, TW; Formal analysis and investigation: NO, YM, YO, AH, SM, KK, KM, HH, TW; Writing – original draft preparation: NO, TW; Writing – review and editing: NO, TW; Funding acquisition: TW; Resources: SM, HH; Supervision: MK.

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Abbreviations

Ab	antibody
ATG16L1	autophagy-related 16-like 1
BM	bone marrow
CD	Crohn's disease
cLPMNC	colonic lamina propria mononuclear cell
DC	dendritic cell
DMSO	dimethyl sulfoxide
ELISA	enzyme-linked immunosorbent assay
GFP	green fluorescence protein
H&E	hematoxylin and eosin
IBD	inflammatory bowel disease
IRF	interferon regulatory factor
MDP	muramyl dipeptide
NF-κB	nuclear factor-kappa B
NOD	nucleotide-binding oligomerization domain
OVA	ovalbumin
PAM	PAM_3CSK4
PGN	peptidoglycan
PI3K	phosphatidylinositol 3-kinase
p-IκBα	phospho-IκBα
PRR	pattern-recognition receptor
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RIPK2	receptor-interacting serine/threonine-protein kinase 2
SAC	*Staphylococcus aureus*
Tg	transgenic
Th	T helper
TLR	Toll-like receptor
TNBS	trinitrobenzene sulfonic acid
UC	ulcerative colitis

Data Availability Statement

The data that support the findings of this study are available from the corresponding author on reasonable request.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this study.

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