The Wnt5a-Ror2 axis promotes the signaling circuit between interleukin-12 and interferon-γ in colitis

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Wnt5a, which regulates various cellular functions in Wnt signaling, is involved in inflammatory responses, however the mechanism is not well understood. We examined the role of Wnt5a signaling in intestinal immunity using conditional knockout mice for Wnt5a and its receptor Ror2. Removing Wnt5a or Ror2 in adult mice suppressed dextran sodium sulfate (DSS)-induced colitis. It also attenuated the DSS-dependent increase in inflammatory cytokine production and decreased interferon-γ (IFN-γ)-producing CD4⁺ Th1 cell numbers in the colon. Wnt5a was highly expressed in stromal fibroblasts in ulcerative lesions in the DSS-treated mice and inflammatory bowel disease patients. Dendritic cells (DCs) isolated from the colon of Wnt5a and Ror2 deficient mice reduced the ability to differentiate naïve CD4⁺ T cells to IFN-γ-producing CD4⁺ Th1 cells. In vitro experiments demonstrated that the Wnt5a-Ror2 signaling axis augmented the DCs priming effect of IFN-γ, leading to enhanced lipopolysaccharide (LPS)-induced interleukin (IL)-12 expression. Taken together, these results suggest that Wnt5a promotes IFN-γ signaling, leading to IL-12 expression in DCs, and thereby inducing Th1 differentiation in colitis.

Wnt5a is a representative ligand that activates the Wnt/β-catenin independent signaling pathway and is one of the most extensively studied ligand in the Wnt family. It plays important roles in the developmental processes of various organs and has postnatal cellular functions. Wnt5a binds to and internalizes its receptor complex, which consists of Frizzled (Fz), a seven transmembrane receptor, and receptor tyrosine kinase-like orphan receptor (Ror) 1 or Ror2, a single transmembrane receptor. Wnt5a binding activates Rho, Rac, protein kinase C, and Jun-N-terminal kinase (JNK), thereby regulating the cytoskeleton, cell migration and polarity, and gene expression.

Mice homozygous for a Wnt5a null allele (Wnt5a⁻/⁻) suffer perinatal lethality because of asphyxia. The embryos are truncated caudally, displaying an inability to extend the embryonic anterior-posterior axis. Furthermore, knockout of Wnt5a down-regulates expression of the pro-apoptotic gene Bax, and promotes expression of the anti-apoptotic gene Bcl-2, which inhibits apoptosis in CD4⁺CD8⁺ double positive thymocytes, suggesting that Wnt5a plays a role in hematopoietic cell development.

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there are no gross abnormalities in the post-natal development of Wnt5a heterozygous (Wnt5a<sup>+/−</sup>) mice, they have a bone-loss phenotype with decreased trabecular bone mass<sup>1</sup>. Aged Wnt5a<sup>+/−</sup> mice have an increased population of B cells and develop myeloid and B-cell leukemia<sup>9</sup>. In addition, it has been suggested that post-natal abnormalities in Wnt5a signaling are involved in inflammatory diseases, as well as cancers<sup>1</sup>.

For instance, expression of Wnt5a and Fz5 can be induced through Toll-like receptors (TLR) stimulated by Mycobacterium tuberculosis (M. tuberculosis) or lipopolysaccharide (LPS) in macrophages, and Wnt5a signaling is involved in producing pro-inflammatory cytokines, such as interleukin (IL)-12 and IL-6<sup>10–12</sup>. Wnt5a has been detected in granulomatous lesions in the lungs of patients with M. tuberculosis, bone marrow macrophages in septic patients, and macrophages accumulated within the intima of atherosclerotic patients<sup>10,15–17</sup>. Thus, these results suggest that Wnt5a released from macrophages in response to inflammatory cues affects macrophages in an autocrine manner to release cytokines. However, the underlying mechanism by which Wnt5a regulates inflammation remains enigmatic. Although purified Wnts are useful tools to better understand the roles of Wnt signaling in the inflammatory responses, it has been reported that recombinant Wnt preparations contain TLR agonists that may lead to inflammatory cytokine production<sup>14</sup>. Therefore, further in vivo studies elucidating the roles of Wnt5a signaling in the immune responses using adult Wnt5a knockout mice are necessary.

Interferon-γ (IFN-γ) is a key immunoregulatory protein that plays a major role in the host innate and adaptive immune responses<sup>15</sup>. IFN-γ is mainly produced in Th cells, which are differentiated from naïve T cells by IL-12 released from antigen-presenting cells, including dendritic cells (DCs) and macrophages<sup>5,16</sup>. Engagement of IFN-γ with its receptor leads to the activation of Janus kinase (JAK) and the phosphorylation of signal transducer and activator of transcription (STAT).<sup>16</sup> STAT1 then translocates into the nucleus where it binds to DNA and initiates the transcription of the STAT1 target genes. IFN-γ also has a priming function and increases inflammatory cytokine production, including tumor necrosis factor-α (TNF-α), IL-6, and IL-12, in response to TLR ligands in DCs and macrophages<sup>5,16</sup>. Thus, it appears that IFN-γ and IL-12 form a signaling circuit between Th cells and antigen-presenting cells.

Here we use dextran sodium sulfate (DSS)-induced colitis in mice as a model for inflammatory diseases and show that disease symptoms were milder in Wnt5a<sup>−/−</sup> and Ror2 conditional knockout mice than control mice. Blocking Wnt5a signaling also reduced the production of pro-inflammatory cytokines in the colon. Finally, we demonstrate that the Wnt5a−Ror2 axis enhances the priming action of IFN-γ to increase TLR-dependent production of IL-12 in DCs, thereby promoting immune responses.

Results

**Wnt5a knockout mice were less susceptible to DSS-induced colitis.** Given that Wnt5a<sup>−/−</sup> mice suffer perinatal lethality<sup>7</sup>, we generated Wnt5a<sup>fl/fl</sup>CAG<sup>Cre</sup>/<sup>Cre</sup> (Wnt5a<sup>ΔΔ</sup>) mice (Figures S1A-C), in which exon 2 of the Wnt5a gene was flanked by loxP sites. These mice were crossed with different Cre-expressing mice, including CAG-Cre/ERT2<sup>7ο</sup> mice, and the offspring were treated with DSS. CAG-Cre/ERT2<sup>7ο</sup> mice show ubiquitous expression of Cre/ERT2 which is activated by administration of tamoxifen<sup>19</sup>. In the Wnt5a<sup>ΔΔ</sup>, CAG-Cre/ERT2<sup>7ο</sup> mice, Wnt5a exon2 was deleted in the colon by administering tamoxifen (Wnt5a<sup>ΔΔ</sup>ΔΔ mice) (Figure S1D). Wnt5a mRNA expression levels varied between Wnt5a<sup>ΔΔ</sup>ΔΔ mouse tissues, but it was remarkably lower in the liver and bone marrow than other tissues (Fig. 1a). In Wnt5a<sup>ΔΔ</sup>ΔΔ mice, Wnt5a mRNA was dramatically decreased in the colon, small intestine, stomach, bone marrow, heart, muscle, and brain; and expression was reduced by half in the liver, spleen, thymus, and lung (Fig. 1b).

DSS (2.5%) was delivered in drinking water to Wnt5a<sup>ΔΔ</sup>ΔΔ mice and their Wnt5a<sup>ΔΔ</sup>ΔΔ littermates. Wnt5a<sup>ΔΔ</sup>ΔΔ mice showed less weight loss than the WT mice following colitis induction (Fig. 2a). DSS caused bleeding in the stools (Fig. 2b) and pasty stools (Fig. 2c) at 5–6 days after its administration. These phenotypes were observed later and to a lesser degree in Wnt5a<sup>ΔΔ</sup>ΔΔ mice (Fig. 2b,c). The overall severity of colitis measured as the disease activity index (DAI), which included scores for body weight loss, occult and gross stool bleeding, and stool consistency, was milder in Wnt5a<sup>ΔΔ</sup>ΔΔ mice than Wnt5a<sup>ΔΔ</sup>ΔΔ mice (Fig. 2d). These results suggested that Wnt5a<sup>ΔΔ</sup>ΔΔ mice were less susceptible to DSS-induced colitis.

The histological patterns in the colon of DSS-fed mice were classified into 4 categories (Figures S2A-D); (1) intact crypts, (2) decreased crypt lesions, (3) monolayer lesions, and (4) ulcer lesions. In WT mice, the frequencies of decreased crypt, monolayer, and ulcer lesions increased gradually over 5 days of DSS administration (Figure S2E). However, the histopathological scores of epithelial damage were reduced in the DSS-fed Wnt5a<sup>ΔΔ</sup>ΔΔ mice compared with Wnt5a<sup>ΔΔ</sup>ΔΔ mice (Fig. 2e and Figure. S2F). Although several Wnt mRNAs, including Wnt1, Wnt4, Wnt5a, Wnt5b, Wnt6, and Wnt11, were expressed in the colon, Wnt5a mRNA was expressed more highly than other Wnt mRNAs, and significantly increased by DSS administration (Fig. 2f). Thus, Wnt5a might be involved in DSS-induced colitis.

**Wnt5a was required for inflammatory cytokine production in the colon.** Many ulcer lesions were observed in the area within a 1 cm distance from the anus in DSS-induced colitis. In the lesions of Wnt5a<sup>ΔΔ</sup>ΔΔ mice, DSS treatment induced increased mRNA expression levels of IL-6, TNF-α, IL-12a, IL-12b, and IFN-γ (Fig. 3a). The increases were suppressed in the colon of Wnt5a<sup>ΔΔ</sup>ΔΔ mice (Fig. 3a). The mRNA levels of IL-17a, IL-10, and transforming growth factor-β31 (TGF-β31) were increased in the colonies of both Wnt5a<sup>ΔΔ</sup>ΔΔ and Wnt5a<sup>ΔΔ</sup>ΔΔ mice by DSS treatment, but their expression levels were not significantly
to the colon was decreased in DSS-induced colitis. However, it is unlikely that T cell recruitment from the mesenteric lymph nodes was significantly decreased by Wnt5a deletion, the frequencies of others were not affected (Figure S3A).

It is thought that intestinal inflammation is caused by an imbalance between the inflammatory response and tolerance. Therefore, the frequencies of CD4+ Th1, Th17, and Treg cells in various tissues were compared in Wnt5aΔ/Δ and Wnt5afl/fl mice (Fig. 3a). Enzyme-linked immunosorbent assay (ELISA) confirmed that DSS-induced production of IL-6 and TNF-α was decreased in the Wnt5aΔ/Δ mouse colon and that production of IL-10, IL-17A, and IL-23A was not changed in Wnt5aΔ/Δ and Wnt5afl/fl mice (Fig. 3b). These results suggested that DSS-induced pro-inflammatory cytokine synthesis in the colon was decreased in Wnt5aΔ/Δ mice compared with control Wnt5afl/fl mice.

Loss of Wnt5a in the bone marrow and intestinal epithelium did not affect DSS-induced colitis. Given that LPS induces Wnt5a expression in bone marrow cells and macrophages and that Wnt5a alters DC responses to LPS, we first speculated that the inhibition of DSS-induced colitis...
Figure 2. Wnt5aCAGΔ/Δ mice show resistance to DSS-induced colitis. (a–c) Wnt5afl/fl (n = 5) and Wnt5aCAGΔ/Δ mice (n = 5) were given DSS for 10 days. As a control, Wnt5afl/fl (n = 3) and Wnt5aCAGΔ/Δ mice (n = 3) were given water. Percent body weight loss (a), bleeding score (b), and stool consistency/diarrhea score (c) of the mice were measured daily. (d) DAI was calculated from the scores of body weight loss, stool consistency, and gross bleeding. (e) The epithelium damage was expressed as the average of the scores of the four pathological classifications. An approximately 1 cm length of the colon from the anus was isolated after Wnt5afl/fl (n = 3) and Wnt5aCAGΔ/Δ mice (n = 3) were administrated with DSS for 7 days, and histologically analyzed. Four histological patterns were classified as follows: intact crypts, decreased crypt lesions, monolayer lesions, and ulcer lesions. The histological patterns were expressed as the frequencies of the four pathological classifications (see Figures S2A–D). (f) WT mice (n = 3) were given DSS for the indicated number of days. Total RNA was extracted from a 1 cm length of the colon from the anus, and the mRNA levels of the indicated Wnts were expressed as fold increases compared with the Wnt5a mRNA level at day 0. The results are shown as means ± SD (a–e) or SE (f). *P < 0.05, **P < 0.01 as calculated by one-way ANOVA (a–e) and by the Student's t-test (f).

**might be caused by the loss of Wnt5a in hematopoietic cells. To address this question, Wnt5afl/fl;Mx-CreTg mice were generated. Mx-Cre mainly deletes genes in hematopoietic cells25. In these mice Wnt5a mRNA expression levels were decreased in the liver, thymus, and bone marrow by the peritoneal administration of polyinosine-polycytosine (pIpC) (Wnt5aΔ/Δ mice) (Fig. 1b). Genomic DNA isolated from the bone marrow of Wnt5aΔ/Δ mice showed efficient digestion of Wnt5a exon2 compared with that of Wnt5afl/fl mice (Figure S4A). However, the Wnt5aΔ/Δ mice had colitis phenotypes that were similar in terms of severity to Wnt5afl/fl mice (Figures S4B and C).**
Villin-Cre depletes genes in the epithelial cells of the intestines. In Wnt5a(+/−);Villin-CreTg mice (Wnt5aΔ/Δ mice), Wnt5a genomic DNA was digested in the colon (Figure S4D). However, the reduction of Wnt5a mRNA in the colon was not clearly observed (Figure S4D), likely because Wnt5a might be mainly expressed in the regions other than epithelium of the colon. The clinicopathological symptoms of DSS-induced colitis in the Wnt5aΔ/Δ mice were unchanged compared with Wnt5a(+/−) mice (Figures S4E and F). Thus, the Wnt5a, if any, released from hematopoietic and intestinal epithelial cells could not be involved in colitis, and Wnt5a produced in other cells may play roles in colitis.
Wnt5a was released primarily from fibroblasts and Ror2 was expressed in DCs in the colon. To examine which cells are the major source of Wnt5a, the colons from mice fed with DSS were analyzed for Wnt5a expression. Wnt5a mRNA was expressed in fibroblasts more highly than epithelial cells and hematopoietic cells, including CD4+ T cells, B220+ B cells, CD11c+ DCs, CD11b+CD11c− macrophages, and γδT cells in the colon, and its expression level was elevated only in the fibroblasts after DSS administration (Figure S5A). In DSS-fed mice, Wnt5a protein and Wnt5a mRNA were not detected in the intact crypt regions of the colon, whereas the protein and mRNA were clearly visible in the mesenchyme of the ulcer lesions (Fig. 5a and Figure S6). Wnt5a-expressing cells were positive for vimentin (a mesenchymal cell marker), but not for F4/80 (a macrophage marker) and CD11c (a DC marker) (Fig. 5b), suggesting that they were fibroblasts. In addition, Wnt5a was still expressed in the mesenchyme of ulcer lesions in Wnt5afl/fl mice but not in Wnt5aCAGΔΔ mice (Fig. 5c). Taken together, these results suggested that Wnt5a secreted from cells other than hematopoietic cells, probably fibroblasts, affects DSS-induced colitis.

Wnt5a expression was then examined in the colon from Crohn’s disease (9 cases) and ulcerative colitis (10 cases) patients. Wnt5a was not detected in the intact crypt regions (E-cadherin positive) (Fig. 5d), but it was clearly detected in the mesenchyme of ulcer lesions (E-cadherin negative) in about half of the inflammatory bowel disease cases (Fig. 5e). As in the mice, the Wnt5a was detected in vimentin-positive cells but not in the cells expressing CD68, a macrophage and monocyte marker (Fig. 5f).

In contrast to Wnt5a, Ror2 mRNA was expressed in hematopoietic cells although it was lower than in fibroblasts (Figure S5B). Among hematopoietic cells, DCs as well as B cells, macrophages, and γδT cells, showed higher Ror2 mRNA expression compared with T cells. However, DSS treatment reduced Ror2 mRNA expression in B and γδT cells (Figure S5B), suggesting that these cells are hard to respond to Wnt5a in DSS-induced colitis. In addition, cell numbers of γδT cells were not changed between Wnt5aCAGΔΔ, Ror2CAGΔΔ, and their control mice irrespective of the presence or the absence of DSS treatment (Figure S7). Taken together with the observations that γδT cells play a role in protections against DSS-induced colitis29,30, DCs might respond to Wnt5a in DSS-induced colitis. In addition, cell numbers of γδT cells were not changed between Wnt5aCAGΔΔ, Ror2CAGΔΔ, and their control mice irrespective of the presence or the absence of DSS treatment (Figure S7). Taken together with the observations that γδT cells play a role in protections against DSS-induced colitis29,30, DCs might respond to Wnt5a in DSS-induced colitis.

Intestinal DC subsets have been identified by the combination and expression levels of specific cell surface antigens and characterized by their functions in immune responses28,30. We classified CD11c+ DCs into three subsets, including CD11c+CD11b+CD103− cells that induce Th1 polarization, CD11c+CD11b−CD70+CXCR1+intermediate cells that induce Th17 polarization, and CD11c+CD11b−CD70+CXCR1high cells that inhibit T cell proliferation29,32. The Ror2 mRNA levels varied among subsets and CD11c+CD11b−CD103− DC cells indeed expressed Ror2 mRNA (Figure S5C), and loss of Wnt5a or Ror2 did not affect total cell numbers of DC subsets in the presence or the absence of DSS treatment (Figure S7). These results suggest that Wnt5a-Ror2 signaling is involved in the activation of intestinal DCs expressing Ror2 rather than their differentiation.

The Wnt5a-Ror2 axis was involved in pro-inflammatory cytokine synthesis in DCs. Intestinal DCs and macrophages play important roles in the regulation of gut homeostasis through induction of helper T cell subsets20,21. Therefore, intracellular signaling cascades for cytokine productions involved in Th1 differentiation were examined in DCs. LPS induced the expression of IL-12α, IL-12b, IL-23a, and IL-6 mRNA in CD11c+ DCs from Wnt5aCAGΔΔ and Ror2CAGΔΔ mice (Fig. 6a,b). However, LPS-induced increases in the expression of these cytokines were reduced in DCs from Wnt5aCAGΔΔ and Ror2CAGΔΔ mice (Fig. 6a,b). Colon CD11c+ DCs induces Th1 differentiation from naive T cells in vitro25,34. The ability of DCs from Wnt5aCAGΔΔ and Ror2CAGΔΔ mice to induce Th1 differentiation was decreased compared with those from Wnt5aCAGΔΔ and Ror2CAGΔΔ mice (Fig. 6c). Similarly, Wnt5a signaling through Ror2 in CD11c+ DCs is required for cytokine expression which induces Th1 differentiation. It is noteworthy that LPS-induced production of IL-12α, IL-12b, IL-23a, and IL-6 mRNA was not suppressed in colon CD11c+ DCs from Wnt5aCAGΔΔ mice (Fig. 6d), which are consistent with the observation that Wnt5aCAGΔΔ mice showed the phenotypes similar to control mice in DSS-induced colitis (see Figures S4B and C).
**Figure 4.** *Ror2*^Δ/Δ^ mice show resistance to DSS-induced colitis. (a) *Ror2*^fl/fl^ (n = 5) and *Ror2*^CAGΔ/Δ^ mice (n = 8) were given DSS for 12 days. The DAI was calculated daily. (b) *Ror2*^fl/fl^ (n = 4) and *Ror2*^CAGΔ/Δ^ mice (n = 8) were given DSS for 13 days. The DAI was calculated daily. (c) The epithelium damage score of *Ror2*^fl/fl^ (n = 3) and *Ror2*^CAGΔ/Δ^ mice (n = 3) administrated with DSS for 7 days was calculated. (d) DSS was administered to *Ror2*^fl/fl^ (n = 7) and *Ror2*^CAGΔ/Δ^ (n = 5) mice for 7 days. As control, *Ror2*^fl/fl^ (n = 3) and *Ror2*^CAGΔ/Δ^ (n = 3) mice were untreated. The mRNA levels of the indicated cytokines were measured by quantitative RT-PCR and expressed as fold increases compared with control *Ror2*^fl/fl^ mice. (e) DSS was administered to *Ror2*^fl/fl^ (n = 3) and *Ror2*^CAGΔ/Δ^ (n = 3) mice for 7 days. As control, *Ror2*^fl/fl^ (n = 3) and *Ror2*^CAGΔ/Δ^ (n = 3) mice were untreated. The supernatants of the lysates of the colon were prepared and the amounts of the indicated cytokines were measured by ELISA. The results are shown as means ± SD (a–c) or SE (d and e). *P < 0.05, **P < 0.01 as calculated by one-way ANOVA (a–c), and the Student's t-test (d and e).
**Figure 5.** DSS causes Wnt5a expression primarily in fibroblasts. (a and b) Tissue sections of the colon at day 10 after administration of DSS were stained with indicated antibodies and DRAQ5. Scale bars, 200 μm (a); 100 μm (b). (c) Tissue sections of the colon isolated from Wnt5aCDGΔ/Δ (left panel) and Wnt5aMxΔ/Δ (right panel) mice at day 10 after administration of DSS were stained with indicated antibodies and DRAQ5. Scale bars, 200 μm. (d) A tissue section of the intact crypt regions in the human colon was stained with indicated antibodies and DRAQ5. Scale bars, 200 μm. (e) Tissue sections of the colon isolated from Crohn’s disease (top panel) and ulcerative colitis (bottom panel) patients were stained with indicated antibodies and DRAQ5. Scale bars, 200 μm. (f and g) Tissue sections of the colon isolated from Crohn’s disease (f) and ulcerative colitis (g) patients were stained with indicated antibodies and DRAQ5. Scale bars, 100 μm.
Figure 6. The Wnt5a-Ror2 axis is involved in pro-inflammatory cytokine synthesis in CD11c+ DCs. (a and b) CD11c+ DCs isolated from the colon of Wnt5a-fl/fl or Wnt5a-CAGΔΔ/ΔΔ mice (a) and Ror2-fl/fl or Ror2-MxΔΔ/ΔΔ mice (b) were stimulated with 100 ng/ml LPS for 4 h and the total RNA was extracted. The mRNA levels of the indicated genes were measured by quantitative RT-PCR and expressed as fold increases compared with expression in control CD11c+ DCs without LPS stimulation. (c) Splenic naïve CD4+ T cells were cocultured with CD11c+ DCs isolated from the colon of Wnt5a-fl/fl (n = 3) or Wnt5a-CAGΔΔ/ΔΔ (n = 3) mice (top panel), or Ror2-fl/fl (n = 3) or Ror2-MxΔΔ/ΔΔ (n = 3) mice (bottom panel) in the presence of 1 μg/ml anti-CD3 antibody for 24 h. The concentrations of IFN-γ in the culture supernatants were measured by ELISA. The results were shown as means ± SD. (d) CD11c+ DCs isolated from the colon of control Wnt5a-fl/fl or Wnt5a-CAGΔΔ/ΔΔ mice were stimulated with 100 ng/ml LPS for 4 h. The mRNA levels of the indicated genes were measured by quantitative RT-PCR and expressed as fold increases compared with expression in control CD11c+ DCs without LPS stimulation. Two indicated conditional knockout mice were used in one experiment (a, b, and d). The results are shown as means ± SE from three independent experiments (a, b, and d). *P < 0.05, **P < 0.01 as calculated by the Student’s t-test (a, b, and d) and by one-way ANOVA (c).
The Wnt/β-catenin pathway in intestinal DCs is required for immunosuppression. Although Wnt5a has been shown to inhibit the β-catenin pathway, mRNA expression levels of Axin2, a target gene of β-catenin signaling, did not increase in CD11c+ DCs from Wnt5aΔCAG/ΔΔ and Ror2ΔMx/ΔΔ mice (Fig. 6a,b). This suggested that the phenotypes induced by Wnt5a and Ror2 deficiency were not due to the activation of the Wnt/β-catenin pathway.

**The Wnt5a-Ror2 axis was also involved in functions of bone marrow-derived DCs.** To examine the roles of Wnt5a signaling in DC functions further, bone marrow cells were isolated from Wnt5aΔCAG/ΔΔ and Ror2ΔMx/ΔΔ mice and incubated with granulocyte macrophage colony-stimulating factor (GM-CSF) for 7 days to generate bone marrow-derived DCs (BMDCs). Loss of Wnt5a or Ror2 suppressed IL-12a, IL-12b, IL-23a, and IL-6 mRNA expression in BMDCs stimulated with LPS (Fig. 7a,b). Ectopic expression of Wnt5a did not affect the basal levels of IL-12a, IL-12b, IL-23a, IL-6, and IL-10 mRNA expression, but did enhance LPS-induced pro-inflammatory cytokine mRNA expression (Fig. 7c). ELISA confirmed that ectopically expressed Wnt5a promotes LPS-induced secretion of IL-6 and IL-12p40 but not that of IL-10 (Fig. 7d). However, Wnt5a did not affect the expression of CD80 and CD86, which indicate DC maturation, in BMDCs (Figure S8), suggesting that Wnt5a signaling did not affect the phenotype of DCs.

To test whether Wnt5a secreted from other cells affects DC functions, bone marrow cells were cocultured with mouse fibroblast cell lines. L cells stably expressing Wnt5a in the presence of GM-CSF for 7 days. However, this coculture experiment failed, because L cells were detached from dishes after 5 days. Instead, HeLaS3 cells expressing Wnt5a (HeLaS3/Wnt5a cells) were used in this experiment (Figure S9A). Knockdown of Wnt5a efficiently reduced both endogenous and exogenous Wnt5a and the phosphorylation of Dishevelled 2 (Dvl2), which indicates the activation of Wnt5a signaling (Figure S9A). HeLaS3 cells neither expressed IL-12 mRNA nor induced IL-6 mRNA in response to LPS regardless of Wnt5a expression levels (Figure S9B). Under these conditions, coculture of bone marrow cells with HeLaS3/Control cells promoted LPS-induced secretion of IL-12p40 and IL-6 in the resultant BMDCs (Fig. 7e), because HeLaS3 cells express Wnt5a endogenously (Figure S9A). Coculture with HeLaS3/Wnt5a cells further enhanced it (Fig. 7e). Enhanced secretions of IL-12p40 and IL-6 were suppressed by knockdown of Wnt5a in these HeLaS3 cells (Fig. 7e). Taken together, these results suggested that the Wnt5a-Ror2 axis between non-hematopoietic cells and DCs promoted pro-inflammatory cytokine production in DCs.

**The Wnt5a-Ror2 axis promoted the priming action of IFN-γ in DCs.** Finally, the mechanism by which Wnt5a promotes inflammation was examined. LPS activates nuclear factor kappa B (NF-κB), JNK, and p38 through TLR4 to induce the expression of IL-12, IL-6, and TNF-α in DCs.7 When control BMDCs from Wnt5aΔβ/β and Ror2Δβ/β mice were stimulated with LPS, inhibitor of kappa B-α (IκB-α) was degraded to induce the nuclear translocation of the NF-κB p65-subunit (NF-κBp65), and JNK and p38 were also phosphorylated (Figures S10A-D). However, these significant changes in the activation of these signaling pathways in BMDCs from Wnt5aΔCAG/ΔΔ and Ror2ΔMx/ΔΔ mice were diminished with Wnt5aΔβ/β and Ror2Δβ/β mice, respectively (Figures S10A-D). Therefore, the Wnt5a-Ror2 axis might promote LPS-dependent cytokine production without affecting the TLR4 signaling pathway directly.

IL-12 from DCs and macrophages plays a critical role in Th1 differentiation and FACS analyses revealed that IFN-γ-producing Th cells in the colon were decreased in Wnt5aΔCAG/ΔΔ mice (see Fig. 3c,d). Therefore, the mechanism underlying how the Wnt5a-Ror2 axis affects the transcription of the IL-12b gene in DCs was examined using the chromatin immunoprecipitation (Chip) assay. LPS induces the recruitment of RNA polymerase II (Pol II) and NF-κBp65 to the IL-12b promoter in macrophages. Pol II and NF-κBp65 were recruited to the IL-12b promoter region by LPS stimulation in BMDCs from Wnt5aΔβ/β and Ror2Δβ/β mice, but their recruitment was diminished in BMDCs from Wnt5aΔCAG/ΔΔ and Ror2ΔMx/ΔΔ mice (Fig. 8a,b). LPS also increased histone H4K8-acetylation (H4K8-Ac) at the IL-12b gene in BMDCs from Wnt5aΔβ/β and Ror2Δβ/β mice, which was reduced in BMDCs from Wnt5aΔCAG/ΔΔ and Ror2ΔMx/ΔΔ mice (Fig. 8a,b). Thus, the Wnt5a-Ror2 axis may enhance LPS-induced transcription of the IL-12b gene in DCs through the formation of Pol II-containing transcription initiation complexes.

IFN-γ primes macrophages for the expression of IL-12b gene.16,40,41 Given that the actions of Wnt5a are similar to the effects of IFN-γ priming, we examined whether the Wnt5a-Ror2 axis affects IFN-γ signaling in DCs. IFN-γ induced the phosphorylation of JAK1 and STAT1 in BMDCs from Wnt5aΔCAG/ΔΔ and Ror2ΔMx/ΔΔ mice, but at a lower level than was observed in control BMDCs from Wnt5aΔβ/β and Ror2Δβ/β mice (Fig. 8c,d). In addition, the IFN-γ-induced recruitment of STAT1 to the IL-12b promoter was decreased in BMDCs from Wnt5aΔCAG/ΔΔ and Ror2ΔMx/ΔΔ mice compared with their control BMDCs (Fig. 8e,f). However, Src activity, which is involved in the Wnt5a and STAT signaling42,43, was not changed in BMDCs from Wnt5aΔCAG/ΔΔ and Ror2ΔMx/ΔΔ mice (Figures S10E and F). Taken together, these results suggested that the Wnt5a-Ror2 axis enhances the priming action of IFN-γ, although the signaling pathway by which Wnt5a activates this action is not known at present.

**Discussion**

Our results support that the Wnt5a-Ror2 axis promotes DSS-induced colitis by enhancing pro-inflammatory cytokine production in the colon. There are two major pathways for Wnt signaling,
The first is the \(\beta\)-catenin dependent pathway, and the second is the \(\beta\)-catenin independent pathway, which is activated by Wnt5a. The \(\beta\)-catenin dependent pathway is activated in intestinal DCs and is required for the secretion of immunosuppressive cytokines\(^{35}\). Although Wnt5a signaling is able to suppress the \(\beta\)-catenin dependent pathway\(^{1,4}\), our results showed that the \(\beta\)-catenin dependent pathway was not activated in CD11c\(^+\) DCs from Wnt5a\(^{CAG/\Delta}\) and Ror2\(^{Mx/\Delta}\) mice. Therefore, the \(\beta\)-catenin independent pathway may regulate intestinal inflammatory responses independently of the \(\beta\)-catenin dependent pathway.

Figure 7. The Wnt5a-Ror2 axis is involved in pro-inflammatory cytokine synthesis in BMDCs. (a) BMDCs isolated from Wnt5a\(^{fl/fl}\) or Wnt5a\(^{CAG/\Delta}\) mice were stimulated with 100 ng/ml LPS for 4 h. The mRNA levels of the indicated cytokines, CD11c, and Axin2 were measured by quantitative RT-PCR, and expressed as fold increases compared with expression levels from control BMDCs without LPS stimulation. (b) BMDCs from Ror2\(^{fl/fl}\) or Ror2\(^{Mx/\Delta}\) mice were stimulated with 100 ng/ml LPS for 4 h. The mRNA levels of the indicated genes were measured by quantitative RT-PCR. (c and d) During the differentiation to BMDCs isolated from WT mice, cells were infected with lentiviruses expressing GFP or Wnt5a at day 3. BMDCs were then stimulated with 10 ng/ml LPS for 4 h (c) or with 10 ng/ml LPS for 8 h (d). The mRNA levels of the indicated genes (c) or the concentrations of the indicated cytokines in the culture supernatants (d) were measured. (e) BMDCs cocultured for 7 days with HeLaS3/Control or HeLaS3/Wnt5a cells transfected with indicated siRNAs were stimulated with 10 ng/ml LPS for 8 h. The concentrations of IL-6 and IL-12p40 in the culture supernatants were measured by ELISA. Two (a and b) indicated conditional knockout mice or two WT mouse (c–e) were used in one experiment. The results are shown as means ± SE from three independent experiments. *\(P < 0.05\), **\(P < 0.01\) as calculated by the Student’s t-test.
Figure 8. The Wnt5a-Ror2 axis enhances LPS-dependent IL-12 synthesis by promoting the priming action of IFN-γ in DCs. (a and b) BMDCs from Wnt5afl/fl or Wnt5aCAGΔ/Δ mice (a) and Ror2fl/fl or Ror2MxΔ/Δ mice (b) were stimulated with 100 ng/ml LPS for 4 h. The recruitment of RNA polymerase II (Pol II), NF-κBp65, and acetylated histone H4 lysine 8 (H4K8-Ac) to the promoters of the IL-12b gene was assessed by ChIP. Representative ChIP results (left panels). The results are expressed as arbitrary units compared with the signal intensities in control cells without LPS stimulation (right panels). (c and d) BMDCs from Wnt5afl/fl or Wnt5aCAGΔ/Δ mice (d) and Ror2fl/fl or Ror2MxΔ/Δ mice (e) were stimulated with the indicated concentrations of IFN-γ for 10 min. The lysates were probed with the indicated antibodies and the results shown are representative of three independent experiments (top panels). The signals detected by indicated antibodies were quantified by NIH image and expressed as arbitrary units (bottom panel). Cropped blots are used. Full scan images of immunoblots are presented in Figures S11 and S12. (e and f) BMDCs from Wnt5afl/fl and Wnt5aCAGΔ/Δ mice (e) and Ror2fl/fl and Ror2MxΔ/Δ mice (f) were stimulated with 30 ng/ml IFN-γ for 4 h. The recruitment of STAT1 to the promoters of the IL-12b gene was assessed by ChIP. Representative ChIP results (top panels). The results are expressed as arbitrary units compared with the signal intensities in control cells without LPS stimulation (bottom panels). Three indicated conditional knockout mice were used in one experiment. The results are shown as means ± SE from three independent experiments. *P < 0.05, **P < 0.01 as calculated by the Student’s t-test.
pathway at least in DCs. It is intriguing to speculate that Wnt signaling may trigger anti- or pro-immune responses through the β-catenin dependent or independent pathway, respectively, in intestinal DCs.

The clinical signs, histological damage, and pro-inflammatory cytokine levels in DSS-induced colitis were suppressed in Wnt5aCAGΔ/Δ and Ror2MIAΔ/Δ mice compared with control mice. In turn, the Wnt5aCAGΔ/Δ and Wnt5aVilΔ/Δ mice showed colitis phenotypes similar to control mice. The latter finding suggests that Wnt5a released from hematopoietic cells (Wnt5aCAGΔ/Δ) or epithelial cells (Wnt5aVilΔ/Δ) in the colon was not involved in DSS-induced colitis. Our results demonstrated that in mice, the basal expression level of Wnt5a in intestinal fibroblasts is much higher than hematopoietic cells or epithelial cells, and that DSS administration induces Wnt5a expression in fibroblasts located in ulcer lesions. The observation that LPS-induced pro-inflammatory cytokines produced from CD11c+ DCs in Wnt5aCAGΔ/Δ and Ror2MIAΔ/Δ mice were decreased but not in Wnt5aVilΔ/Δ mice also support that hematopoietic cells are not the primary source of Wnt5a in the colon. In addition, our human studies show that Wnt5a was clearly observed in vimentin-positive cells, but not macrophages, in the ulcer lesions of patients with ulcerative colitis and Crohn’s diseases. Taken together, these results suggest that fibroblasts might be the primary source of Wnt5a at least in the colon during inflammatory bowel disease.

It has been reported that Wnt5a can act in a paracrine manner on neighboring cells. For example, the Wnt5a-Ror2 axis between osteoblast-lineage cells and osteoclast precursors enhances osteoclastogenesis, while Ror2 deficiency in osteoclast precursors leads to impaired osteoclastogenesis. In addition, the Wnt5a-Fz8/Flamingo axis functionally maintains HSCs in their niche through interactions between N-cadherin-positive osteoblasts, which express Wnt ligands including Wnt5a, and long-term HSCs that express Fz8 and Flamingo.

Methods

Animals. The protocols used for all animal experiments in this study were approved by the Animal Research Committee of Osaka University, Japan (No. 21–048–1). All animal experiments were carried out according to the guidelines for the care and use of experimental animals of Osaka University. Details of Wnt5aβ/β mouse line generation are described in Supplementary Methods. Ror22/2 mice were generated as described.
CAG-Cre/ERT2 mice and Villin-Cre mice were purchased from Jackson Laboratory. To treat the mice, a stock solution of tamoxifen (4-hydroxytamoxifen; Sigma-Aldrich, St. Louis, MO) in ethanol (150 mg/ml) was diluted in corn oil to 15 mg/ml. The tamoxifen suspension (0.1 ml) was administered to adult Wnt5afl/fl or Ror2fl/fl;CAG-Cre/ERT2 mice 4 weeks of age and relevant control Wnt5afl/fl or Ror2fl/fl mice three times at two day intervals to induce the activation of the Cre-ERT2 recombinase and to remove the floxed Wnt5a or Ror2 coding region. Mx-Cre expression was induced by the intraperitoneal injection of 300 μg plPC (Sigma-Aldrich) six times at two day intervals to remove the floxed Wnt5a or Ror2 coding region. The primers used for genotyping in this study were listed in Supplementary Table S1.

**Induction of colitis.** DSS was administered to eight to nine weeks old male mice in their drinking water at a final concentration of 2.5% (w/v) to induce colitis. Animals were observed daily for weight, stool consistency, and the presence of gross blood in feces and at the anus. Detailed evaluation of colitis is described in Supplementary Methods.

**Preparation of colon extraction.** After 7 days administration of DSS, 2 cm length of colon from anus was isolated, and homogenized in homogenization buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM phenylmethylsulfonylfluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin) by down homogenizer (15 strokes). After the homogenization, 1% Nonidet P-40, 1% deoxycholic acid and 0.1% sodium dodecyl sulfate (SDS) were added to the homogenates. The homogenates were sonicated 6 times for 15 sec by Ultra S homogenizer (TAITEC, Nagoya, Japan), incubated at 4°C with rotation for 1 h and then centrifuged at 20,000 g for 10 min. The supernatants were used in ELISA.

**Patients and tissues of inflammatory bowel diseases.** Tissue samples from 20 patients who underwent surgery for Crohn’s disease and ulcerative colitis at Osaka University Hospital from 2011 to 2013 were examined. Histological specimens were fixed in 10% formalin and routinely processed for paraffin embedding. Paraffin-embedded specimens were stored in the dark room in the Department of Pathology at Osaka University Hospital at room temperature and cut into 4 μm thick sections at the time of staining. The study was approved by the ethics review board of Graduate School of Medicine, Osaka University (No. 13455).

**Isolation of lamina propria DCs, bone marrow-derived DCs (BMDCs), and lymphocytes.** Isolations of lamina propria DCs, BMDCs, and lymphocytes were performed as described by Ueda Y. et al., Kayama H. et al., and Kusu T. et al., respectively. Details of experimental procedures are described in Supplementary Methods.

**Coculture of naïve CD4 T cells with lamina propria DCs.** Coculture of naïve CD4 T cells with lamina propria DCs were performed as described by Atarashi K. and Kayama H. et al. Details of experimental procedures are described in Supplementary Methods.

**General experimental procedures.** General experimental procedures are described in Supplementary Methods.

**Statistical analysis.** The experiments in each figure were performed three to four times, and differences between control and experimental groups were evaluated using the Student’s t-test and a one-way ANOVA with a Bonferroni test for multiple group comparison. A P value < 0.05 was considered a significant difference.

**Others.** Quantification data was calculated based on at least three blots or gels from different experiments. All the gels were run under the same experimental condition as detailed in the Supplementary Methods.

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**Author Contributions**

A.S. designed the experiments, carried out the mouse and cell level experiments, and wrote the manuscript. H.K. and K.T. designed the immunological experiments and H.K. carried out the FACS analysis. K.S. and S.M. carried out the histological and immunohistochemical experiments. H.K. and H.H. generated the Wnt5a floxed mice. Y.M. generated the Ror2 floxed mice. S.N. and E.M. provided human IBD samples and analyzed them. A.K. designed experiments and wrote the manuscript.

**Additional Information**

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