Rag2-deficient IL-1 Receptor Antagonist-deficient Mice Are a Novel Colitis Model in Which Innate Lymphoid Cell-derived IL-17 Is Involved in the Pathogenesis

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Abstract: Il1rn−/− mice spontaneously develop arthritis and aortitis by an autoimmune mechanism and also develop dermatitis by an autoinflammatory mechanism. Here, we show that Rag2−/−Il1rn−/− mice develop spontaneous colitis with high mortality, making a contrast to the suppression of arthritis in these mice. Enhanced IL-17A expression in group 3 innate lymphoid cells (ILC3s) was observed in the colon of Rag2−/−Il1rn−/− mice. IL-17A-deficiency prolonged the survival of Rag2−/−Il1rn−/− mice, suggesting a pathogenic role of this cytokine in the development of intestinal inflammation. Although IL-17A-producing T cells were increased in Il1rn−/− mice, these mice did not develop colitis, because CD4+Foxp3+ regulatory T cell population was also expanded. Thus, excess IL-1 signaling and IL-1-induced IL-17A from ILC3s cause colitis in Rag2−/−Il1rn−/− mice in which Treg cells are absent. These observations suggest that the balance between IL-17A-producing cells and Treg cells is important to keep the immune homeostasis of the colon.

Keyword: Colitis, IL-1, IL-17, innate lymphoid cells, regulatory Tcells

Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the gastrointestinal tract, characterized by both intestinal inflammation and mucosal tissue destruction [15, 35]. Although the etiopathogenesis of IBD is not completely understood, an excessive host immune response against intestinal commensal microbiota is suggested to be involved in the disease development.
of Th17 cells in intestinal inflammation is controversial [24]. Recent studies have shown that the majority of IL-17A is derived from innate-like or innate immune cells in inflammatory sites, especially in gut mucosal tissues [4]. Indeed, intestinal inflammation in many of IBD models develops in immunodeficient mice lacking adaptive immunity [2, 26, 33]. Moreover, the pathogenic role of innate immune cell-derived IL-17A has been directly demonstrated using Helicobacter hepaticus (H. hepaticus)-infected 129SvEvRag2−/− mice [2].

γδ T cells and group 3 innate lymphoid cells (ILC3s) share many characteristics with Th17 cells. These cells can produce IL-17A and IL-22 immediately after stimulation without further differentiation, because these cells constitutively express orphan nuclear receptor RORγt and signature cytokine receptor IL-23R [31, 32]. IL-1β and IL-23 stimulation induces IL-17A in the absence of TCR signaling in these cells [3, 31]. Thus, both types of cells contribute to host defense in the early phase after infection.

γδ T cells are shown to induce intestinal inflammation in colitis models [6, 23, 25]. ILC3s are a recently identified leukocyte lineage marker negative (Lin−) and RORγt+ cell population with CD4+ Thy1.2hiSca-1hi-c-kithi markers [30], and ILC3-derived IL-17A is suggested to be involved in the pathogenesis of colitis. In H. hepaticus-infected colitis model, IL-17A production from ILC3s causes colonic inflammation in response to bacteria-induced IL-23 [2]. In addition, IL-17A production from ILC3s can induce spontaneous colitis in Tbx21−/− Rag2−/− mice [26]. However, the in vivo mechanism of IL-17A production from these cells and that of disease development remain to be elucidated.

IL-1β is a proinflammatory cytokine that causes various inflammatory responses in both innate and adaptive immunity. IL-1β induces the expansion and the maintenance of Th17 cells [14] and also activates γδ T cells and ILCs to produce IL-17A in collaboration with IL-23 [29, 32]. IL-1 receptor antagonist (IL-1Ra, gene symbol: Il1rn) is an endogenous IL-1 inhibitor, which competes with IL-1α and IL-1β for IL-1 receptor (IL-1R) binding. We previously showed that IL-1Ra-deficient (Il1rn−/−) mice on BALB/cA background spontaneously develop inflammatory diseases, such as arthritis [13], aortitis [19], and dermatitis [22], indicating that strict regulation of IL-1 activity is critical for immune homeostasis. Using cytokine-deficient mice and adoptive cell transfer, we demonstrated that arthritis and aortitis develop by a T cell-dependent autoimmune mechanism whereas dermatitis is caused by a T cell-independent autoinflammatory mechanism [12, 19, 22]. Moreover, TNF-α deficiency restrains the development of both arthritis and dermatitis, whereas IL-17A deficiency does not suppress dermatitis development. Thus, Il1rn−/− mice are a unique inflammatory disease model in which both adaptive and innate immune cell-mediated inflammatory responses are observed.

In this report, we show that Rag2−/−Il1rn−/− mice develop spontaneous severe colitis with high mortality, making a contrast to the suppression of arthritis in these mice. Enhanced IL-17A expression in ILC3s was observed in the colon of Rag2−/−Il1rn−/− mice and IL-17A deficiency prolonged the survival of Rag2−/−Il1rn−/− mice, suggesting a pathogenic role of this cytokine in the exacerbation of intestinal inflammation. Because Rag2−/−Il1rn−/− mice, in which Foxp3+ Treg cells were absent and ILC3s were expanded, developed colitis, but Il1rn−/− mice, in which Treg cell population was increased, did not develop colitis in spite of the expansion of IL-17A-producing T cells, it is suggested that the balance between IL-17A-producing cells and Treg cells is important for the homeostasis of intestinal immunity.

Materials and Methods

Mice

BALB/cA genetic background mice were used throughout this study. BALB/cA and nu/nu mice were purchased from CLEA Japan. Il1rn−/− mice were produced as described [11]. nu/nuIl1rn−/− mice were produced by crossing Il1rn−/− mice with nu/nu mice (CLEA Japan). Rag2−/− mice were a gift from Dr. Sakaguchi (Osaka University, Japan) [10]. Rag2−/− mice were crossed to Il1rn−/− mice to generate Rag2−/−Il1rn−/− mice. IIl7a−/−Il1rn−/− mice were generated as described [21] and were crossed to Rag2−/−Il1rn−/− mice to generate IIl7a−/−Rag2−/−Il1rn−/− mice. Age-matched and sex-mixed mice were used for all experiments, except Fig. 4, in which only females were used. All mice were kept under specific pathogen-free conditions in environmentally controlled clean rooms at the Center for Experimental Medicine and Systems Biology, The Institute of Medical Science, The University of Tokyo, and Institute for Biomedical Sciences, Tokyo University of Science. All experiments were approved by the institutional ethical committees for animal experiments and the com-
mittees for gene manipulation experiments.

**Histologic analysis of the colon**

Colon tissues were removed from mice at 10-weeks of age, and paraffin sections were made and stained with hematoxylin and eosin.

**Isolation of LP cells in the colon**

LP cells were isolated from the colon as described [34] with modifications. Briefly, colon tissues were removed from mice and cut into 1 cm pieces, and incubated in RPMI medium (Sigma, St. Louis, MO, USA) containing 10% heat-inactivated FCS and 5 mM EDTA to remove epithelial cells. The remaining tissues were further digested with RPMI medium containing 10% FCS, 5 mM EDTA, and 200 U/ml collagenase (Sigma). LP cells were then layered on a 75% Percoll gradient (Sigma), and after centrifugation at 2,200 rpm for 20 min, cells were collected at the 40%/75% Percoll interface.

**Flow cytometry**

Intracellular cytokine staining was performed as described previously [16] after stimulation with 50 ng/ml PMA (Sigma), 500 ng/ml ionomycin (Sigma), and 2 µM monensin (Sigma) for 5 h. For cell surface molecule staining, cells were first treated with anti-mouse CD16/CD32 mAbs (2.4G2, purified from hybridoma culture supernatant) in staining buffer (HBSS containing 2% FCS and 0.1% sodium azide) to block FcR binding, then cells were stained with the following antibodies: PE-anti-mouse γδ TCR mAbs (GL3; BioLegend), PE-anti-mouse Ly6A/E (Sca-1) mAbs (D7; eBioscience, San Diego, CA, USA), PE/Cy7-anti-mouse CD4 mAbs (GK1.5; BioLegend), APC-anti-mouse CD8α mAbs (53–6.7; BioLegend), APC-anti-mouse CD335 (NKp46) mAbs (29A1.4; BioLegend), APC/Cy7-anti-mouse CD3ε mAbs (145-2C11; BioLegend), Pacific Blue-anti-mouse CD90.2 (Thy1.2) mAbs (30-H12; BioLegend), Pacific Blue-anti-mouse CD117 (c-kit) mAbs (2B8; BioLegend). For the analysis of leukocyte lineage marker negative cells, cells were stained with the following antibodies: FITC-anti-mouse CD4 mAbs (GK1.5; BioLegend), FITC-anti-mouse CD8α mAbs (53–6.7; BioLegend), FITC-anti-mouse γδ TCR mAbs (GL3; eBioscience), FITC-anti-mouse CD11b mAbs (M1/70; BioLegend), FITC-anti-mouse CD11c mAbs (HL3; BD Permingen), FITC-anti-mouse Gr-1 mAbs (RB6-8C5; BioLegend), FITC-anti-mouse B220 mAbs (RA-3-6B2; BioLegend). Then, cells were fixed with 4% paraformaldehyde. For intracellular cytokine staining, cells were treated with a permeabilization buffer (0.1% saponin [Sigma] in staining buffer), and incubated with the following antibodies: FITC-anti-mouse IFN-γ mAbs (XMGl.2; eBioscience), FITC-anti-mouse IL-10 mAbs (JES5-16E3; BioLegend), and Pacific Blue-anti-mouse IL-17A mAbs (TC11-18H10.1; BioLegend). Intracellular staining was performed with Foxp3 Staining Kit according to the manufacturer’s instructions (eBioscience) with the following antibodies: PE-anti-mouse Foxp3 mAbs (FJK-16s; eBioscience) and APC-anti-mouse RORγt mAbs (AFKJS-9; eBioscience). 7-Aminoactinomycin D (Sigma) was used for staining dead cells. Cells were analyzed on a FACS CantoII system (Becton Dickinson, Franklin Lakes, NJ, USA) and data were analyzed with FlowJo software (Tree Star).

**Real-time PCR**

Total RNA from anal and colon was extracted with Sepasol reagent (Nacalai Tesque, Kyoto, Japan) according to the manufacturer’s instructions and was denatured in the presence of an oligo dT primer and then reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative real time PCR was performed with a SYBR Green qPCR kit (Invitrogen, Carlsbad, CA, USA) and an iCycler system (Bio-Rad, Hercules, CA, USA) with the sets of the following primers:

- **Gapdh**
  - 5′-TTC ACC ACC ATG GAG AAG GC-3′ and 5′-GGC ATG GAC TGT GGT CAT GA-3′;
- **Il17a**
  - 5′-TTT AAC TCC CTT GGC GCA AAA-3′ and 5′-CTT TCC CTC CGC ATT GAC AC-3′;
- **Il17f**
  - 5′-TGC TAC TGT TGA TGT GAC-3′ and 5′-AAT GCC CTG GTT TGA TGT GAC-3′;
- **Il12a**
  - 5′-GAA CTG GCA AAA GGA TGG TGA-3′ and 5′-TGT GGG TTG TTG ACC TCA-3′;
- **Il1b**
  - 5′-AGT CTC TTC TTC TCG CTC-3′ and 5′-GCC CAT GAT GAT CTC TCT CAA-3′;
- **Il22**
  - 5′-TGG TTA CTG GAA GCA AGG CAT TGA TGT GAC-3′ and 5′-ATG GCC CAT GAT CTC TCT CAA GTG AT-3′;
- **Il11a**
  - 5′-TGG GGA GGA GAC GAC GAC TAT TAT AAC-3′ and 5′-GAC GAC TCG CCG CAC CAG CTA GTG-3′;
- **Il12p40**
  - 5′-TCG CTT ATC CCC CAA CAA CCC CTC CCA-3′ and 5′-GCC GAT GAT CTC TCT CAA GTG AT-3′;
- **Il12p35**
  - 5′-CTG CTA CCT CACC CCA-3′;
- **Il1a**
  - 5′-GAA TCC TGC TGG TGT GAC-3′ and 5′-GAC TCT CCG CAC CAG CTA GTG-3′;
- **Il1rn**
  - 5′-AAA TGG ACT TGG CTT TCC TGT TGT TGC CAC TCA GAT-3′ and 5′-ATT GAT CTC TCT CCA GTG AT-3′;
- **Il11b**
  - 5′-AGA TCC TGT GGA ATC TCT GCA-3′ and 5′-AGT CCA ACA AGT CTC TCT CCA GTG AT-3′;
- **Il12p40**
  - 5′-GCA GAA GGA CAC CAG CTA GTG-3′ and 5′-GCC GAT GAT CTC TCT CAA GTG AT-3′;
- **Il12p35**
  - 5′-CTG TGG TGT GAC-3′ and 5′-GAC TCT CCG CAC CAG CTA GTG-3′;
- **Il1rn**
  - 5′-AAA TGG CAG TCG CTA GTC TCT CTA ATT-3′ and 5′-AGA TCC TCA GGC CCA-3′.

Cells were analyzed on a FACS CantoII system (Becton Dickinson, Franklin Lakes, NJ, USA) and data were analyzed with FlowJo software (Tree Star).
G-3' and 5'-GCA GAG TCT CGC CAT TAT GAT TC-3'; Il23p19 5'-ATG CTG GAT TGC AGA GCA GTA-3' and 5'-CAC TTG GTG TGT TCG TAC GA-3'; Il6 5'-GAG GAT ACC ACT CCC AAC AGA CC-3' and 5'-AAG TGC ATC ATC TTA GGT GTT TGC TAC GA-3'; Cxcl1 5'-GCA ACA TGT GGA ACT CTG A-3' and 5'-AGT GAT TT-3' and 5'-GCC TCC CTC TCA TCA GTT CT-3' and 5'-CAC CCA CTC A-3'; Cxcl2 5'-CCA ACC ACC AGG CTA CAG G-3' and 5'-GAC GTC AAA AGA CAG TAG AGA GC-3' and 5'-GCC TCA CAC TCA AGC TCT G-3'; 5'-GCG TCA CAC TCA AGC TCT G-3'; 5'-ATG CTG GAT TGC AGA GCA GTA-3'.

**Statistics**

Unless otherwise specified, all results are shown as the mean and the SEM. Unpaired Student’s t tests, Mann-Whitney’s U tests, or logrank tests were used to statistically analyze the results. Differences were considered significant at P<0.05.

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**Results**

**nu/null1rn**<sup>−/−</sup> mice spontaneously develop colitis

To analyze the roles of IL-1 system in innate immunity, we crossed **Il1rn**<sup>−/−</sup> mice on BALB/cA background into Foxn1<sup>−/−</sup> (nu/nu) mice that lack functional thymus-derived T cells. These **nu/null1rn**<sup>−/−</sup> mice spontaneously developed severe colitis associated with anorectal prolapse (Fig. 1A). Sixteen mice out of 20 showed anorectal prolapse at 12-weeks of age. Both females and males showed similar symptoms. Colonic wall thickening and shortening of the large intestine were observed in the prolapsed mice (Fig. 1B). Histological analysis revealed mucosal thickening, crypt distortion, and infiltration of a large number of inflammatory cells in the mucosa and submucosa of the large intestine, including the anus, rectum, and colon (Fig. 1C). Infiltration of inflammatory cells was also observed in the colon of non-prolapsed **nu/null1rn**<sup>−/−</sup> mice (Fig. 1D), indicating that the spontaneous development of colonic inflammation is a characteristic of **nu/null1rn**<sup>−/−</sup> mice.

**Rag2**<sup>−/−</sup>**Il1rn**<sup>−/−</sup> mice spontaneously develop colitis

Unlike αβ T cells, γδ T cells can develop extrathymically in **nu/nu** mice [1, 27]. To investigate whether or not extrathymically developed-γδ T cells induce colitis in **nu/null1rn**<sup>−/−</sup> mice, we crossed **Il1rn**<sup>−/−</sup> mice to **Rag2**<sup>−/−</sup> mice in which γδ T cells also do not develop. **Rag2**<sup>−/−</sup>**Il1rn**<sup>−/−</sup> mice still developed severe colitis with marked colonic shortening and inflammation (Fig. 2A). The signs of colitis, such as diarrhea, bloody stool, perianal mucus excretion, and anorectal prolapse, appeared after 5-weeks of age, and increased in severity over time (Fig. 2B), with weight loss (Fig. 2C). WT, **Il1rn**<sup>−/−</sup>, and **Rag2**<sup>−/−</sup> mice did not show any symptoms of colitis. About half of them succumbed as early as 11-weeks of age (Fig. 2D). These observations suggest that innate immune cells, but not γδ T cells, are responsible for the spontaneous development of colitis in **Rag2**<sup>−/−</sup>**Il1rn**<sup>−/−</sup> mice.

**IL-17A expression is enhanced in the inflammatory sites of **Rag2**<sup>−/−</sup>**Il1rn**<sup>−/−</sup> mice**

Intestinal macrophages produce IL-1β in response to commensal bacteria [29], and IL-1 can induce own IL-1 expression [5, 11] that can form a positive feedback loop. To examine the effect of IL-1Ra-deficiency on IL-1 expression in the colon, we analyzed the colonic IL-1 expression in **Il1rn**<sup>−/−</sup> and **Rag2**<sup>−/−</sup>**Il1rn**<sup>−/−</sup> mice, and compared with WT and **Rag2**<sup>−/−</sup> mice, respectively. The expression of **Il1a** and **Il1b** was not changed in **Il1rn**<sup>−/−</sup> mice compared to WT mice, indicating that IL-1Ra deficiency per se does not affect IL-1 production. However, **Il1a** and **Il1b** expression were significantly augmented in the perianal tissue of **Rag2**<sup>−/−**Il1rn**<sup>−/−</sup> mice (Fig. 3A). Furthermore, **Il17a** and **Il17f** expression as well as **Il22** and **Il6** expression were also enhanced in **Rag2**<sup>−/−**Il1rn**<sup>−/−</sup> mice (Fig. 3B). The expression of chemokines, such as **Cxc1l** and **Cxc1l2** that induce neutrophil recruitment, was also significantly enhanced in **Rag2**<sup>−/−**Il1rn**<sup>−/−</sup> mice (Fig. 3B). The expression of other inflammatory cytokines, including **Ifng**, **Il4**, **Il12p35**, **Il12p40**, **Il23p19**, and **Tnfa** was not changed in **Rag2**<sup>−/−**Il1rn**<sup>−/−</sup> mice compared with **Rag2**<sup>−/−</sup> mice (Fig. 3B). These results indicate that IL-1Ra-deficiency-caused excess IL-1 signaling results in the increased IL-17A production in **Rag2**<sup>−/−**Il1rn**<sup>−/−</sup> mice.

**IL-17A exacerbates colonic inflammation in **Rag2**<sup>−/−</sup>**Il1rn**<sup>−/−</sup> mice**

To examine whether the increased IL-17A expression in the colon causes the development of colitis in **Rag2**<sup>−/−**Il1rn**<sup>−/−</sup> mice, we have generated **Il17a**<sup>−/−**Rag2**<sup>−/−**Il1rn**<sup>−/−</sup> mice by intercrossing **Rag2**<sup>−/−**Il1rn**<sup>−/−</sup> mice with **Il17a**<sup>−/−</sup> mice. Although the disease signs were not completely
disappeared, Il17a−/−Rag2−/−Il1rn−/− female mice significantly restored from weight loss (Fig. 4A) and high mortality (Fig. 4B). We did not determine the effect of the IL-17A-deficiency in the mortality of Rag2−/−Il1rn−/− male mice. These results suggest that excess IL-17A production exacerbates colitis and promotes mortality in Rag2−/−Il1rn−/− mice.

Innate lymphoid cells are major source of IL-17A in the colon of Rag2−/−Il1rn−/− mice
Consistent with local mRNA expression, IL-17A-producing cells were increased in the colonic lamina propria (cLP) of Rag2−/−Il1rn−/− mice (Fig. 5A), and the majority of IL-17A-expressing cells were CD4−, CD8−, and γδ TCR− cell population (Fig. 5B). These cells were also negative for leukocyte lineage marker (Lin) (Fig. 5C), and showed RORγt+, Sca-1+, Thy-1+, NKp46−, c-
kit− phenotype (Fig. 5D), consistent with the characteristics of ILC3s [2]. These findings suggest that ILC3s are responsible for the increased IL-17A production in Rag2−/−Il1rn−/− mice.

Absence of Treg cells and expansion of ILC3s cause colitis in Rag2−/−Il1rn−/− mice

IL-1β is crucial for intestinal IL-17A production in both innate and adaptive immune cells [29, 31]. Consistent with this, colonic IL-17A expression was increased in Il1rn−/− immunocompetent mice compared with WT mice (Fig. 3B, Fig. 5A and 5B). The majority of IL-17A producers in cLP was γδ T cells, and Th17 and CD4+CD8−γδ TCR− cells also produced IL-17A. However, these Il1rn−/− mice did not develop colitis, suggesting that the IL-17A levels were not enough to induce colitis or some suppressor cells might suppress the development of colitis, or both.

Then, we examined Treg cells in these mice. CD4+Foxp3+ Treg cells were greatly increased in cLP of Il1rn−/− mice compared with that of WT mice (Fig. 6A and 6B). On the other hand, no Foxp3+ cells were found in both Rag2−/− mice and Rag2−/−Il1rn−/− mice. These results suggested that Treg cells suppress the development of colitis in Il1rn−/− mice. Although Treg cell-derived immunosuppressive cytokines, such as IL-10 and TGF-β, can mediate the suppression of wide range of immune responses and prevent experimental colitis [18], no significant increase of these cytokines was observed in both Il1rn−/− and Il1rn−/−Rag2−/− mice (Fig. 6C), and Treg cells in Il1rn−/− mice did not express IL-10 (Fig. 6A). Instead, the ILC3 components that express both

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**Fig. 2.** Rag2−/−Il1rn−/− mice spontaneously develop colitis. (A) Macroscopic analysis of the colon of WT, Il1rn−/−, Rag2−/− and Rag2−/−Il1rn−/− mice. (B) Incidence of rectal prolapse of Rag2−/−Il1rn−/− (open diamonds, n=20), and Rag2−/−Il1rn−/− mice (filled squares, n=11). (C) Body weight of Rag2−/−Il1rn−/− (n=8, female) and Rag2−/−Il1rn−/− mice (n=9, female) at 8-weeks of age. (D) Survival rate of Rag2−/−Il1rn−/− (open diamonds, n=20) and Rag2−/−Il1rn−/− mice (filled squares, n=11). Data are representative of two independent experiments.
Fig. 3. IL-17A expression is enhanced in the inflammatory sites of Rag2\(^{-/-}\)Il1rn\(^{-/-}\) mice. (A and B) The expression of mRNAs for IL-1α and IL-1β (A), and IFN-γ, IL-4, IL-17F, IL-22, IL-6, IL-12p40, IL-12p35, IL-23p19, TNF-α, CXCL1, and CXCL2 (B), in the perianal tissue of WT, Il1rn\(^{-/-}\), Rag2\(^{-/-}\), and Rag2\(^{-/-}\)Il1rn\(^{-/-}\) mice. Data are normalized to GAPDH in perianal tissue (3 to 4 mice per each group) and shown as mean ± SEM. **: \(P < 0.01\) vs. WT mice, †: \(P < 0.05\), ††: \(P < 0.01\), †††: \(P < 0.001\) vs. Rag2\(^{-/-}\) mice. Data are representative of two independent experiments.
Thy 1.2 and Sca-1 were robustly increased in cLP of Rag2−/− and Rag2−/−Il1rn−/− mice (Fig. 6D). IL-1Ra expression in Rag2−/− mice was similar to that in WT mice (Fig. 6E), suggesting that the increase of ILC3 population in Rag2−/− mice is independent of IL-1 systems. These results suggest that both the expansion of IL-17A-producing ILC3s and the absence of Treg cells in Rag2−/−Il1rn−/− mice are responsible for the development of colitis in these mice.

Discussion

In this study, we showed that IL-1Ra-deficiency in nu/nu mice as well as in Rag2−/− mice results in the development of spontaneous colitis. In Rag2−/−Il1rn−/− mice, IL-17A-producing ILC3s were increased in colonic LP and IL-17A deficiency recovered their mortality, suggesting that IL-17A-producing ILC3s are responsible for the exacerbation of colitis in these mice.

Recent studies have suggested the importance of innate immune cells in the pathogenesis of colitis. γδ T cells drive intestinal inflammation in both innate and T cell-induced colitis [6, 23, 25], and activated γδ T cells are increased in the intestinal tissue in IBD patients [20, 36]. Thus, we first thought that γδ T cells might be important for the development of colitis in nu/nullIl1rn−/− mice. However, the severe colitis was also developed in Rag2−/−Il1rn−/− mice, indicating that colitis develops without γδ T cells at least in Rag2−/−Il1rn−/− mice, although this does not necessarily exclude the possibility that γδ T cells are involved in the development of colitis in nu/nullIl1rn−/− mice. In Rag2−/−Il1rn−/− mice, IL-17A production was enhanced in inflamed tissues, and the majority of colonic IL-17A was produced by ILC3s. These colitogenic ILC3s previously found in two colitis mouse models: H. hepaticus-infected 129SvEvRag2−/− mice [2] and Tbx21−/−Rag2−/− mice [26]. Elevated IL17A mRNA expression is also observed in the intestinal CD3−CD56− innate lymphoid cells of IBD patients [9].

IL-1β is crucial for the activation of intestinal IL-17A-producing cells. Recent studies have shown that commensal bacteria specifically induce IL-1β in LP macrophages [29], and induce IL-1R expression on IL-17A-producing γδ T cells [7]. Importantly, innate immune cells can produce IL-17A by the action of IL-1β and IL-23 without TCR signaling. Indeed, IL-17A-producing cells, which consist of mostly γδ T cells and small proportions of Th17 cells and ILC3s, were increased in Il1rn−/− mice compared to WT mice even without inflammation. We found that ILC3s are the major IL-17A producer in Rag2−/−Il1rn−/− mice, in which Th17 and γδ T cells are absent, and IL-17A-deficiency can significantly prolong their survival. Thus, these observations suggest that excess IL-1β-induced ILC3-derived IL-17A participates in the development of colitis in Rag2−/−Il1rn−/− mice. However, because IL-17A-deficiency did not completely suppress the development of colitis although it extended survival, IL-17A is not the principal mediator of the inflammation. Probably, excess IL-1β signaling itself causes inflammation in the

Fig. 4. IL-17A exacerbates colonic inflammation in Rag2−/−Il1rn−/− mice. (A) Body weight of Il17a+/+Rag2−/−Il1rn−/− (n=4, female) and Il17a−/−Rag2−/−Il1rn−/− mice (n=4, female) at 10-weeks of age. Data are shown as mean ± SEM. (B) Survival rate of Il17a+/+Rag2−/−Il1rn−/− (closed squares, n=10, female) and Il17a−/−Rag2−/−Il1rn−/− mice (opened circles, n=8, female). *, P<0.05.
RAG2 KO-IL-1RA KO MICE DEVELOP COLITIS

intestine in an autoinflammatory manner, and IL-17A exacerbates the disease.

Treg cells are one of important regulatory cells in the immune system [17], and can suppress γδ T cell-derived IL-17A-induced intestinal inflammation [25, 37]. We found that IL-17A-producing ILC3s are increased in Rag2<sup>−/−</sup>Il1rn<sup>−/−</sup> mice compared with Il1rn<sup>−/−</sup> mice, correlated with the absence of Treg cells in Rag2<sup>−/−</sup>Il1rn<sup>−/−</sup> mice. These observations suggest that Treg cells suppress IL-17A production and/or differentiation of ILC3s in Il1rn<sup>−/−</sup> mice. Treg cells may also suppress the development of colitis in Il1rn<sup>−/−</sup> mice in which relatively large

Fig. 5. Innate lymphoid cells are major source of IL-17A in the colon of Rag2<sup>−/−</sup>Il1rn<sup>−/−</sup> mice. (A and B) IL-17A- and IFN-γ-expressing cells in colonic lamina propria (cLP) of WT, Il1rn<sup>−/−</sup>, Rag2<sup>−/−</sup>, and Rag2<sup>−/−</sup>Il1rn<sup>−/−</sup> mice were analyzed by FACS. Isolated cLP cells were stimulated with PMA and ionomycin for 5 h and stained for intracellular IL-17A and IFN-γ. All live lymphocytes are shown. (C and D) Characterization of IL-17A-expressing cells in cLP of Rag2<sup>−/−</sup>Il1rn<sup>−/−</sup> mice. All live lymphocytes are shown. Cells were stained by the mixture of antibodies against lymphocyte lineage markers (Lin: CD4, CD8, γδ TCR, CD11b, CD11c, Gr-1, and B220) and intracellular IL-17A. Numbers refer to percent cells in IL-17<sup>+</sup> cells (C). Lin<sup>−</sup>IL-17A<sup>+</sup> cells were stained by specific antibodies (black line) and isotype controls (gray line) (D). Data are representative of two independent experiments.
Fig. 6. Absence of Treg cells and expansion of ILC3s cause colitis in Rag2−/−Il1rn−/− mice. (A and B) Foxp3- and IL-10-expressing cells in cLP of WT, Il1rn−/−, Rag2−/−, and Rag2−/−Il1rn−/− mice were analyzed by FACS. Isolated cLP cells were stimulated with PMA and ionomycin for 5 h and stained for intracellular IL-10 and intranuclear Foxp3. All live lymphocytes are shown.

(C and E) mRNA expressions of IL-10 and TGF-β (C), and secretion form of IL-1Ra (E) in the perianal tissue of WT, Il1rn−/−, Rag2−/−, and Rag2−/−Il1rn−/− mice. Data are normalized to GAPDH in the perianal tissue (3 to 4 mice per each group) and shown as mean ± SEM. (D) FACS analysis for ILC cell population in cLP of WT, Il1rn−/−, Rag2−/−, and Rag2−/−Il1rn−/− mice. All live lymphocytes (top) and Lin−CD45.2+ cells are shown (bottom). Data are representative of two independent experiments.
number of IL-17 producers is detected. Because recent studies suggested that CD4 T cells and ILCs mutually regulate their activity by competing for a cytokine niche [28], it is possible that the expansion of ILC3s in Rag2<sup>−/−</sup> Il1rn<sup>−/−</sup> mice is caused by the loss of competition with neighboring Th17 cells for limited spaces and resources and not the defect of suppression by Treg cells.

In summary, we showed that excess IL-1 signaling-induced IL-17A production from expanded ILC3s and the absence of Treg cells promote the spontaneous development of colitis in Rag2<sup>−/−</sup> Il1rn<sup>−/−</sup> mice, suggesting the importance of the balance between IL-17A-producing cells and Treg cells in the regulation of intestinal immune homeostasis. Il1rn<sup>−/−</sup> mice are unique because these mice spontaneously develop autoimmune arthritis and aortitis and also develop autoinflammatory skin lesions. In this report, we have further demonstrated that this strain is also useful as a colitis model in which innate immune cells play a critical role.

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