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Exacerbation of non-steroidal anti-inflammatory drug-induced enteropathy in C-C chemokine receptor type 7-deficient mice

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Key words

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Abstract

Background and Aim: Non-steroidal anti-inflammatory drugs (NSAIDs) induce intestinal enteropathy and the pathophysiology is related to immune-mediated mechanisms. We aimed to investigate the role of C-C chemokine receptor type 7 (CCR7) which regulates immune cell migration in NSAID-induced enteropathy.

Methods: Injury of the small intestine was evaluated 24 h after the subcutaneous injection of indomethacin in CCR7-deficient (*Ccr7*^{-/-}) and wild-type (WT) mice. The cellular profile and cytokine production in intestinal cells were analyzed. Indomethacin-induced enteropathy was evaluated in mice adoptively transferred with CD103⁺ dendritic cells (DCs) from *Ccr7*^{-/-} or WT mice.

Results: Indomethacin induced more severe intestinal injury in *Ccr7*^{-/-} mice than in WT mice. The major inflammatory cytokines were not increased and the proportion of regulatory T cells following indomethacin injection was not decreased in *Ccr7*^{-/-} mice compared with WT mice. The expression of interleukin (IL)-22 binding protein (IL-22BP), which inhibits IL-22 activity, was significantly higher in CD103⁺ DCs from *Ccr7*^{-/-} mice than those from WT mice. Mice adoptively transferred with CD103⁺ DCs isolated from *Ccr7*^{-/-} mice exhibited more severe intestinal injury following indomethacin injection compared with those adoptively transferred with CD103⁺ DCs of WT mice. *Ccr7*^{-/-} mice injected with indomethacin showed a significant reduction in regenerating islet-derived 1 (*Reg1*) mRNA expression, which is regulated by IL-22, in intestinal epithelial cells.

Conclusions: C-C chemokine receptor type 7 deficiency exacerbated NSAID-induced enteropathy in association with an altered phenotype of CD103⁺ DCs that produces IL-22BP. CCR7 contributes to protect the small intestine from NSAID-induced mucosal injury.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are used widely for their strong pain-relieving activity, which occurs via inhibition of cyclooxygenase enzymatic activity and prostaglandin production.¹ Although NSAIDs are effective, 60–70% of regular NSAID users are reported to suffer from enteropathy.² NSAIDs induce the gastrointestinal mucosal damage through their inhibition of mucus production and promotion of hyperpermeability.³ NSAIDs also cause mitochondrial dysfunction in mucosal epithelia and increase apoptosis via a cyclooxygenase-independent mechanism.⁴ In addition, immune-mediated mechanisms were reported to be involved in the pathophysiology of NSAID-induced mucosal injury.^{5–7} Our previous study showed that NSAID-induced enteritis was more severe in mice that were deficient of Peyer's patches (PPs) owing to the impairment of anti-inflammatory CD103⁺ dendritic cells (DCs) in the mesenteric

lymph nodes (MLN). Decrease of CD103⁺ DCs lead to reduction of interleukin (IL)-10 producing CD4⁺ T cells in the lamina propria of the small intestine.⁸ Previous studies showed that CD103⁺ DCs migrated from the intestine under the influence of CC-chemokine receptor (CCR) 7 and that the proportion of CD103⁺ DCs in the MLN has been shown to be decreased in CCR7-deficient (*Ccr7*^{-/-}) mice.⁹ CCR7 is reported to be expressed in various immune cells and regulates migration of mucosal immune cells to egress from resident tissues to the draining lymph nodes via afferent lymphatics.¹⁰ CCR7 was identified on the surface of activated DCs and naive T cells, and subsequent studies reported that CCR7 was re-expressed on activated T cells, guiding their exit from peripheral tissues and their entry to afferent lymphatics.^{11,12} The ligands for CCR7 are chemokine (C-C motif) ligand (CCL)19 and CCL21; their main source is the stromal cells in lymphoid tissues and lymphatic endothelial cells in the

peripheral tissues.^{13–15} CCR7 deficiency exacerbates intestinal inflammation by the impairment of recruitment of activated lymphocytes from the intestine in a tumor necrosis factor (TNF)-driven murine ileitis model.¹⁶ This report showed increased activity of T-helper (Th) 1 and decrease of regulatory T (Treg) cells in the MLN.¹⁶ To date, there were no reports regarding the NSAID-induced mucosal injury in *Ccr7*^{-/-} mice, which have diminished the population of CD103⁺ DCs. There are a variety of mechanisms for the dysregulation of mucosal immune function for the induction of intestinal inflammation other than dysregulated Th1/Th17 and Treg function: IL-22 has mucosal barrier function and dysregulation of IL-22 induces intestinal inflammation.¹⁷ The underline mechanism of NSAID-induced enteropathy has not been clarified. This study sought to clarify the mechanism of NSAID-induced enteritis in relation to CCR7 and CD103⁺ DCs by using *Ccr7*^{-/-} mice. Here, we report an acceleration of intestinal injury by the impairment of IL-22 affected by CD103⁺ DCs in *Ccr7*^{-/-} mice.

Methods

Mice. C57BL/6 wild-type (WT) mice were purchased from Japan Clea (Tokyo, Japan) and *Ccr7*^{-/-} mice (C57BL/6 background) were generated by Prof. Martin Lipp (Max-Delbrück-Center for Molecular Medicine), as reported previously.¹⁸ Deficiency of CCR7 was confirmed by genotyping and flow cytometry (Fig. S1). Male mice between 8 and 10 weeks of age were used in this study. All mice were kept under specific pathogen free conditions in an environmentally controlled clean room at the Institute of Experimental Animal Sciences of the Osaka University Graduate School of Medicine. The Institutional Committee on Animal Research approved all experimental protocols.

Induction of indomethacin-induced enteropathy.

Enteropathy was induced by subcutaneous injection with indomethacin (10 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) in mice. The mice were euthanized 24 h after the injection of indomethacin. The tissues of the small intestine (SI) were opened along the anti-mesenteric attachment and were examined under a stereomicroscope for ulcerative lesions. The ulcerated area (in square millimeters) in the SI was measured by tracing the ulcers under $\times 20$ magnification using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Histology of the ulcers in the small intestine was evaluated by HE staining.

Isolation of mucosal immune cells and epithelial cells.

Mononuclear cells from the MLN were isolated by mechanical dissociation.¹⁷ SI-lamina propria (LP) mononuclear cells and epithelial cells in the SI were collected as described previously.^{17,19} Briefly, after the removal of the PPs from the SI, the tissues were washed with phosphate buffered saline and cut into 1-cm pieces. The pieces were incubated with 0.5 mM ethylenediaminetetraacetic acid (EDTA), agitated slowly, and the supernatants were collected and processed to separate epithelial cells. Epithelial cells were obtained from the interface between the 20% and 40% layers of a discontinuous Percoll gradient (GE Healthcare, Waukesha, WI, USA). Precipitated pieces for the

SI-LP cells were isolated by enzymatic dissociation using collagenase (Wako, Osaka, Japan) and DNase (Sigma-Aldrich) with stirring at 37°C; subsequently, mononuclear cells were obtained from the interface between the 40% and 75% layers of a discontinuous Percoll gradient.

Quantitative real-time polymerase chain reaction.

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and complementary DNA was synthesized using the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) for *Il4* (Mm00445259-m1), *Il10* (Mm1288386-m1), *Ifng* (Mm9999071-m1), *Il17a* (Mm00439618-m1), *Il22* (Mm01226722-m1), *Il22ra2* (or IL22 binding protein [bp], Mm01192969-m1), *Foxp3* (forkhead box p3, Mm-00475162-m1), regenerating islet-derived (*Reg*) 1 (Mm00485651-m1), *Reg3g* (Mm00441127-m1), *Muc1* (Mm00449604-m1), *Muc13* (Mm00495397-m1), *Cldn1* (Mm00516701-m1), and *beta-actin* were performed using Thunderbird Probe qPCR Mix (Toyobo Life Science, Osaka, Japan) and QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). Primers were obtained from Applied Biosystems and the expression of *beta-actin* was used as an endogenous control.

Flow cytometric analysis. The following reagents were used to stain the murine mononuclear cells: Pacific Blue (PB)-labeled anti-CD4, phycoerythrin (PE)-labeled anti-CD103, Alexa Fluor 488-labeled anti-Foxp3, (BD Biosciences, San Jose, CA), PE-labeled anti-CCR7 (eBioscience, San Diego, CA, USA), fluorescein isothiocyanate-labeled anti-CD8a (eBioscience), PB-labeled anti-CD11c, phycoerythrin/Cy7-labeled CD11b (Biolegend, San Diego, CA, USA), allophycocyanin (APC)-labeled IL-22BP (R & D systems). Intracellular IL-22BP was stained using Cytofix/Cytoperm (BD Biosciences), and Foxp3 was stained using mouse Foxp3 Buffer Set (BD Biosciences) in accordance with the manufacturer's instructions. These samples were subjected to flow cytometric analysis using a FACS Canto II (BD Biosciences). The data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Western blot analysis. The procedures of protein extracts were described previously.²⁰ Epithelial cells of the small intestine were lysed with a lysis buffer [1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 1× protein inhibitor cocktail (Nacalai Tesque, Kyoto, Japan), phosphate-buffered saline; pH 7.4]. After incubation on ice for 15 min, the lysate was centrifuged at 10 000 g for 15 min at 4°C. The protein content of the supernatants was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). For Western blot analysis, the primary antibodies anti-mouse Reg1, Reg3G, Muc1 (Abcam, Cambridge, UK), and GAPDH (Cell Signaling Technology, Danvers, MA, USA) were used. The donkey anti-rabbit immunoglobulin G-horseradish peroxidase (Santa Cruz Biotechnology, Dallas, TX, USA) was used as a secondary antibody.

Adoptive transfer of mesenteric lymph nodes mononuclear cells. The CD103⁺ DCs isolated from the MLN of either indomethacin-treated WT or *Ccr7*^{-/-} mice were sorted by flow cytometry; subsequently, CD103⁺ DCs (2×10^5 cells) were intraperitoneally injected to WT mice. At 24 h after the adoptive transfer, indomethacin (10 mg/kg body weight) was subcutaneously injected. After a further 24 h, all mice were euthanized.

Statistical analysis. The results are expressed as the mean and standard error. Difference was tested by ANOVA in the groups of three and data between the two groups were compared by Student's *t*-test. The differences were considered statistically significant for *P* values of < 0.05 .

Results

***Ccr7*^{-/-} mice develop severe indomethacin-induced enteropathy.** Neither WT nor *Ccr7*^{-/-} mice spontaneously exhibited apparent macroscopic and microscopic inflammation in

the SI. When indomethacin was subcutaneously injected into WT and *Ccr7*^{-/-} mice, these mice showed multiple ulcers in the SI. Although the number of ulcers per SI did not differ significantly, the size of average ulcerated area was significantly larger in the *Ccr7*^{-/-} mice than in the WT mice (Fig. 1a–d). The total ulcerated area in the SI of *Ccr7*^{-/-} mice was significantly larger than that of WT mice (Fig. 1e).

Cellular profile of the immune cells in WT and *Ccr7*^{-/-} mice after indomethacin administration.

Prostaglandins have been shown to protect from mucosal injury and are suppressed by NSAIDs.¹ However, the prostaglandin E2 levels in the SI-LP were not significantly different between WT and *Ccr7*^{-/-} mice (Fig. S2). We next investigated the cellular profile of MLN and SI-LP in WT and *Ccr7*^{-/-} mice in the presence and absence of indomethacin. The proportion of CD11c⁺ cells in the MLN was significantly higher in *Ccr7*^{-/-} mice than in WT mice both before and after the administration of indomethacin (Fig. 2a). There was no significant difference in the proportion of CD4⁺ and CD11b⁺ cells in the MLN between WT and *Ccr7*^{-/-}

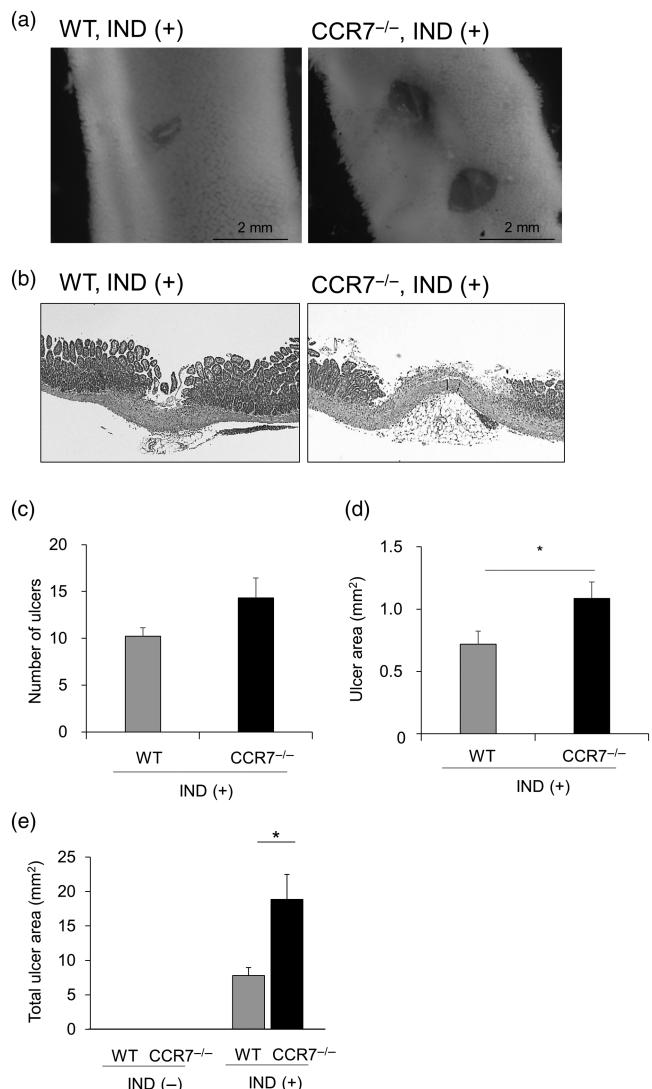


Figure 1 WT and *Ccr7*^{-/-} mice showed multiple ulcers in the small intestine after subcutaneous injection of indomethacin. Representative pictures of ulcers in the small intestine after subcutaneous injection of indomethacin to WT and *Ccr7*^{-/-} mice (a). Representative HE pictures of the ulcers in the small intestine after subcutaneous injection of indomethacin to WT and *Ccr7*^{-/-} mice (b). The number of ulcers per mouse in WT (■) and *Ccr7*^{-/-} mice (■) after injection of indomethacin (c). The average size of the ulcerated area in WT (■) and *Ccr7*^{-/-} mice (■) after injection of indomethacin (d). Summations of the ulcerated area in the small intestine per mouse in both WT (■) and *Ccr7*^{-/-} mice (■) (e). The data are expressed as the mean + standard error of 8–10 mice per group. IND, Indomethacin. **P* < 0.05 by Student's *t*-test in the comparison between WT and *Ccr7*^{-/-} mice.

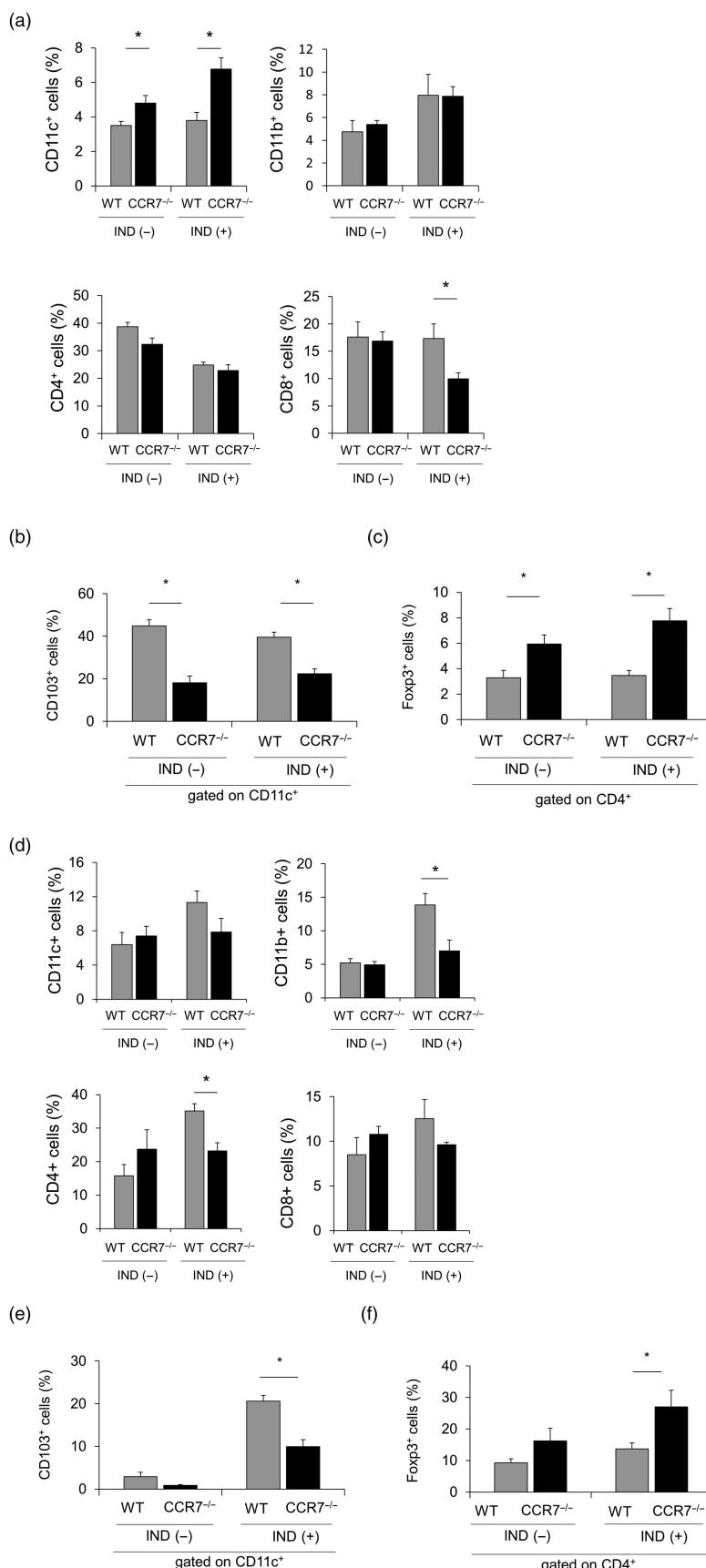


Figure 2 The immune cell profile of mononuclear cells in MLN and SI-LP of WT and *Ccr7^{-/-}* mice. Proportion of CD4⁺, CD8⁺, CD11c⁺, and CD11b⁺ cells in MLN of WT (■) and *Ccr7^{-/-}* mice (■) injected with or without indomethacin (a). The proportion of CD11c⁺ CD103⁺ cells in MLN of WT (■) and *Ccr7^{-/-}* mice (■) injected with or without indomethacin (b). The proportion of CD4⁺ Foxp3⁺ cells in MLN of WT (■) and *Ccr7^{-/-}* mice (■) injected with or without indomethacin (c). The proportion of CD4⁺, CD8⁺, CD11c⁺, and CD11b⁺ cells in SI-LP of WT (■) and *Ccr7^{-/-}* mice (■) injected with or without indomethacin (d). The proportion of CD103⁺ cells in SI-LP CD11c⁺ cells of WT (■) and *Ccr7^{-/-}* mice (■) injected with or without indomethacin (e). The proportion of Foxp3⁺ cells in SI-LP CD4⁺ cells of WT (■) and *Ccr7^{-/-}* mice (■) injected with or without indomethacin (f). IND, Indomethacin. The data are expressed as the mean + standard error of 4–6 mice per group. **P* < 0.05 by Student's *t*-test in the comparison between WT and *Ccr7^{-/-}* mice separately in IND (-) and IND (+) groups.

mice in the presence and the absence of indomethacin (Fig. 2a). We next investigated the proportion of CD4⁺ Foxp3⁺ T cells, which are known to be induced by CD103⁺ DCs and to have immune suppressive function.^{21,22} The proportion of CD103⁺ cells in the CD11c⁺ DCs in the MLN was significantly lower in *Ccr7*^{-/-} mice than in WT mice before and after the administration of indomethacin (Fig. 2b). In contrast, the proportion of CD4⁺ Foxp3⁺ cells in the MLN was significantly higher in *Ccr7*^{-/-} mice than in WT mice before and after indomethacin administration

(Fig. 2c). In the SI-LP, there was no difference in the CD4⁺, CD8⁺, CD11c⁺, and CD11b⁺ proportions between WT and *Ccr7*^{-/-} mice in the absence of indomethacin administration; however, the proportions of CD4⁺ and CD11b⁺ were significantly decreased in *Ccr7*^{-/-} mice compared with WT mice after the administration of indomethacin (Fig. 2d). In the SI-LP of *Ccr7*^{-/-} mice, similarly to MLN, the proportion of CD103⁺ DCs was decreased, whereas that of CD4⁺ Foxp3⁺ T cells was increased compared with WT mice (Fig. 2e,f). These results refuted the

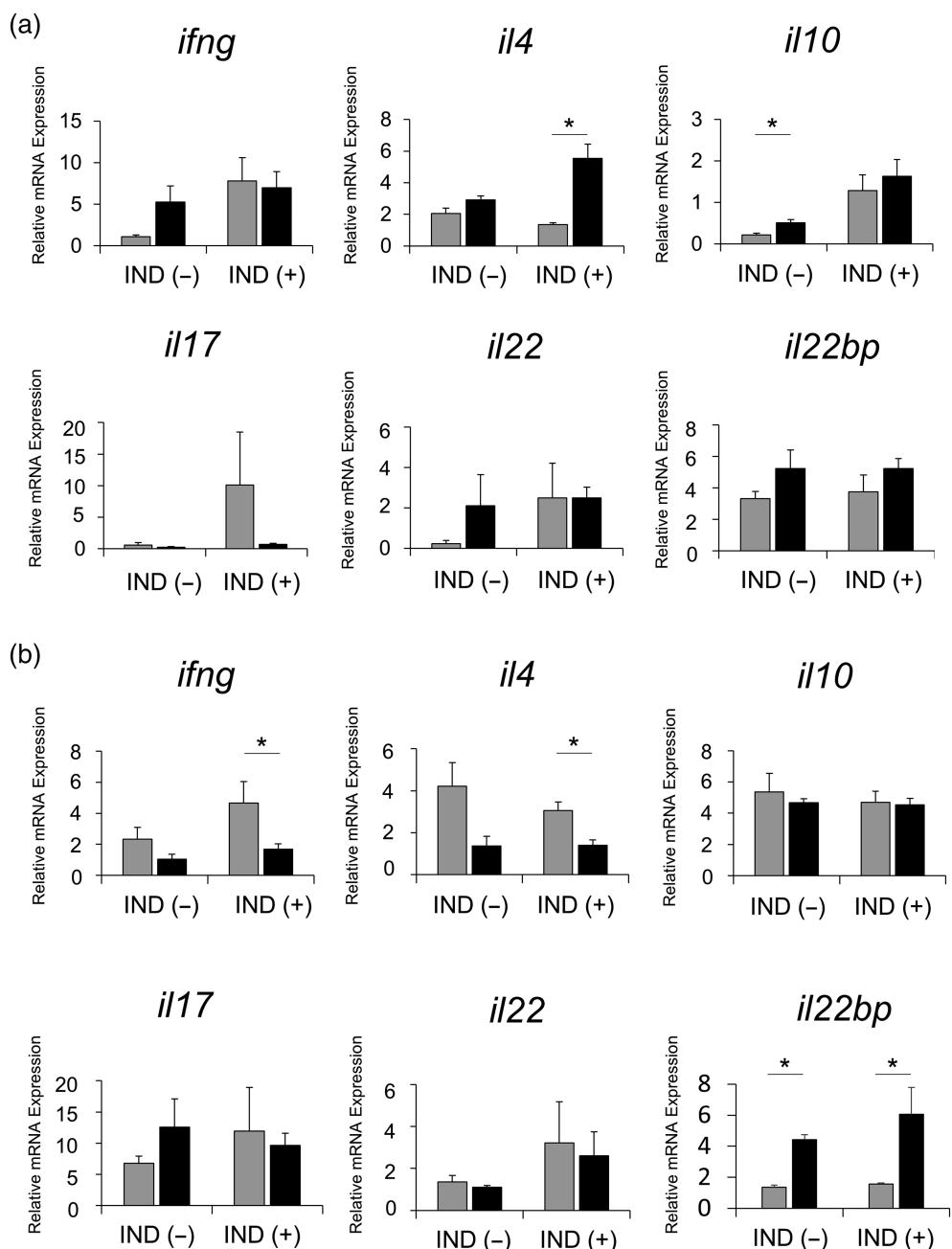


Figure 3 qRT-PCR analysis of cytokine profile of mononuclear cells in MLN and SI-LP. Cytokine mRNA expression in MLN of WT mice (■) and *Ccr7*^{-/-} mice (■) injected with indomethacin was analyzed by qRT-PCR after injection of indomethacin (a). Cytokine mRNA expression in SI-LP was analyzed (b). The data are expressed as the mean + standard error of three to five mice per group. IND, indomethacin. *P < 0.05 by Student's t-test.

possibility that the exacerbation of intestinal injury in *Ccr7*^{-/-} mice was caused by a decrease in regulatory T cell population.

Cytokine production in the mononuclear cells of MLN and SI-LP in WT and *Ccr7*^{-/-} mice after administration of indomethacin. We then analyzed cytokine production in MLN and SI-LP of WT and *Ccr7*^{-/-} mice after indomethacin injection. In the MLN, the expression of *il4* mRNA was significantly higher in *Ccr7*^{-/-} mice than in WT mice, while there was no difference in the expression of *ifng*, *ttnfa*, *il10*, *il17*, *il22*, and *il22bp* mRNA between the two groups (Fig. 3a). The cytokine profile in the SI-LP showed that the expressions of *ifng*, *il4*, *il1b*, and *il6* mRNA were significantly lower in *Ccr7*^{-/-} mice than in WT mice (Fig. 3b, Fig. S3), and there was no difference in the expression of *ttnfa*, *il10*, *il17*, and *il22* mRNA between the two groups (Fig. 3b). Thus, no increase of major inflammatory cytokines and decrease of anti-inflammatory cytokine were observed in *Ccr7*^{-/-} mice. The expression of *Il22bp* was found to be significantly higher in the SI-LP of *Ccr7*^{-/-} mice than in WT mice both in the presence and absence of indomethacin (Fig. 3b).

Increased expression of IL-22BP in CD103⁺ CD11c⁺ cells in *Ccr7*^{-/-} mice. We then investigated the source of IL-22BP in the SI-LP of *Ccr7*^{-/-} mice. When SI-LP cells were divided into four subsets based on the expression of CD11c and CD103, IL-22BP was highly expressed in CD103⁺ CD11c⁺ cells (CD103⁺ DCs) relative to other subsets in both WT and *Ccr7*^{-/-} mice (Fig. 4a). In addition, the population of CD103⁺ DCs

expressing IL-22BP was significantly higher in *Ccr7*^{-/-} mice than WT mice (Fig. 4a,b).

Decreased expression of regenerating islet-derived 1 in *Ccr7*^{-/-} mice.

IL-22 serves to induce mucin and regenerating proteins that comprise the potent barrier of the intestinal mucosa.²³ When we analyzed the expression of mucin and regenerating gene proteins in the SI epithelial cells of WT and *Ccr7*^{-/-} mice, the expression of *Regl* mRNA was significantly lower in *Ccr7*^{-/-} mice than in WT mice before and after the administration of indomethacin (Fig. 5a). In addition, the protein expression of Regl was lower in *Ccr7*^{-/-} mice than in WT mice with indomethacin injection (Fig. 5b). We next analyzed the fecal microbiota of WT and *Ccr7*^{-/-} mice. Alpha diversity of the fecal samples was lower in *Ccr7*^{-/-} mice that were administered indomethacin was lower than in WT mice (Fig. S4A) and beta diversity of *Ccr7*^{-/-} mice administered indomethacin was different from other groups (Fig. S4B). The components of the intestinal bacterial flora in *Ccr7*^{-/-} mice administered indomethacin revealed alteration of the microbial profile at the phylum level compared with other groups: *Bacteroidetes* was decreased, whereas *Firmicutes* and *Proteobacteria* were increased in *Ccr7*^{-/-} mice administered indomethacin (Fig. S4C).

Adoptive transfer of CD103⁺ dendritic cells isolated from *Ccr7*^{-/-} mice showed more severe intestinal injury than transfer of the cells isolated from WT mice.

We next performed adoptive-transfer experiments of CD103⁺ DCs isolated from either WT or *Ccr7*^{-/-} mice to WT

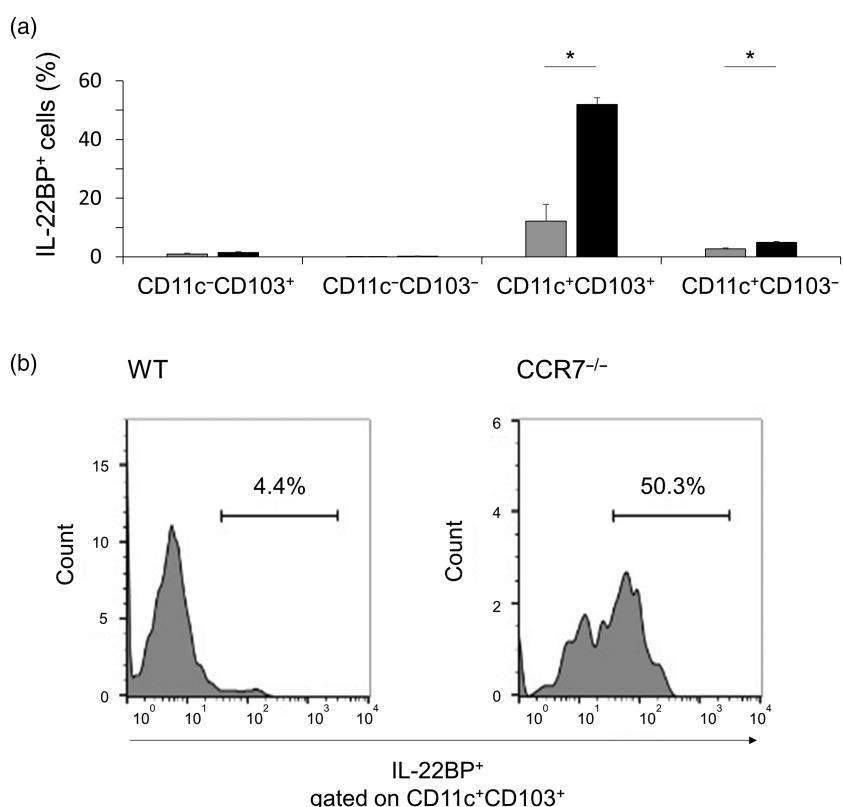


Figure 4 Flow cytometric analysis of cells expressing IL-22BP. The proportion of IL22BP⁺ cells in each subset of positive/negative cells for CD11c and CD103 in WT (■) and *Ccr7*^{-/-} mice (■) that were injected with indomethacin (a). Representative histogram of IL-22BP positive cells gated in CD103⁺ DCs in mice administered indomethacin (b). The data are expressed as the mean + standard error of three mice per group (a). Representative pictures from three independent experiments are shown (b). *P < 0.05 by Student's t-test.

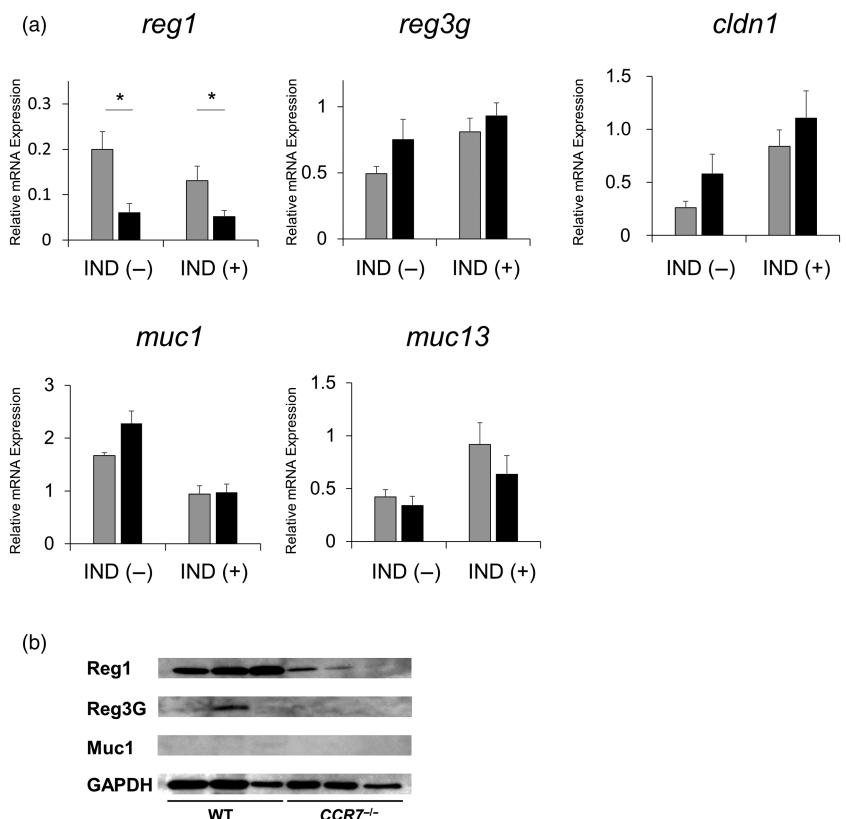


Figure 5 mRNA expression of regenerating and mucin genes in the epithelial cells of the small intestine. The mRNA expression of *Reg1*, *Reg3g*, *Cldn1*, *Muc1*, and *Muc13* in WT (■) and *Ccr7*^{-/-} mice (■) injected with/without indomethacin (a). The data are expressed as the mean + standard error of 6 mice per group. IND, Indomethacin. **P* < 0.05 by Student's *t*-test. The protein expression of *Reg1*, *Reg3G*, *Muc1*, and GAPDH in the small intestinal epithelial cells of WT and *Ccr7*^{-/-} mice with indomethacin-injection (b).

mice and enteropathy was induced in these mice by indomethacin injection. The ulcerated area of the mice transferred with CD103⁺ DCs derived from *Ccr7*^{-/-} mice was significantly larger than that of the mice adoptively transferred with CD103⁺ DCs derived from WT mice or WT mice without adoptive transfer (Fig. 6a,b). The expression of *Il22bp* mRNA in the mononuclear cells of SI-LP was significantly higher in mice adoptively transferred with CD103⁺ DCs from *Ccr7*^{-/-} mice than those adoptively transferred with WT cells (Fig. 6c).

Discussion

We demonstrated that SI enteropathy induced by indomethacin was exacerbated in *Ccr7*^{-/-} mice in association with increased IL-22BP production in CD103⁺ DCs. CCR7 plays an important role for the homing of naïve T cells and DCs in the secondary lymphoid organs.^{24,25} Several reports have linked CCR7 and intestinal inflammation: In a mouse model of TNF-driven Crohn's-like ileitis, ileitis was exacerbated in *Ccr7*^{-/-} mice. The severity of ileitis was associated with a decreased population of CD103⁺ DCs and CD4⁺ Foxp3⁺ CD25⁺ Tregs and with increased IFN-γ producing-Th1 cells.¹⁶ In another model of colitis using SCID (severe combined immunodeficiency) mice adoptively transferred with CD4⁺CD25⁻ naïve T cells, CD4⁺ Tregs isolated from *Ccr7*^{-/-} mice did not ameliorate colitis.²⁶ Therefore, we initially speculated that deterioration of enteropathy was related to a decrease in Treg cells and an increase in Th1 cells. However, the proportion of Foxp3⁺ Treg cells was higher in *Ccr7*^{-/-} mice than WT mice (Fig. 2f). In addition, Th1 cytokines and IL-10 did not skew toward induction of inflammation in *Ccr7*^{-/-} mice (Fig. 3). These

results clearly showed that increased Th1 cells and decreased Treg cells were not the major cause of the enteropathy exacerbation.

In this study, we found increased IL-22BP production in CD103⁺ DCs isolated from *Ccr7*^{-/-} mice which is related to exacerbation of enteropathy. IL-22 is a member of the IL-10 cytokine family and targets at cellular outer-body barriers.²⁷ IL-22 induces the production of mucus-associated proteins, such as MUC1 and MUC13 and regenerating islet-driven protein family members in intestinal epithelial cells.^{28,29} IL-22 acts via a transmembrane receptor complex consists of two different subunits: IL-22 receptor (R) 1 and IL-10R2.³⁰ IL-22 also binds to IL-22BP and this binding prevents the interaction of IL-22 with IL-22R1 or IL-22R2. IL-22BP is a secreted IL-22R encoded by an IL-22R1-independent gene. The affinity between IL-22 and IL-22BP is 20- to 1,000-fold higher than that between IL-22 and IL-22R1.^{23,31} IL-22BP is expressed in the epithelium and various immune cells.³¹⁻³³ In DCs, IL-22BP is expressed on immature DCs and the expression of IL-22BP is decreased in association with cell maturation.^{31,34,35} We observed an increase in *Il22bp* expression in CD103⁺ DCs of *Ccr7*^{-/-} mice. Because CCR7 controls the maturation of intestinal DCs,³⁶ increased IL-22BP expression may be related to the insufficient maturation of CD103⁺ DCs in *Ccr7*^{-/-} mice. The importance of IL-22BP has been reported in patients with inflammatory bowel disease (IBD) and enteritis mouse models. High levels of IL-22BP in DCs, CD4⁺ T cells, and eosinophils are reported in the inflamed lesions of patients with IBD.^{37,38} The delivery of IL-22BP *in vivo* showed abrogation of the IL-22-mediated protection in a model of chemical-induced colitis.³⁹ Transfer of WT CD4⁺ CD25⁻ CD45Rb^{high} T cells into *Rag1*^{-/-} or *Rag1*^{-/-} *Il22bp*^{-/-} mice resulted in severe intestinal

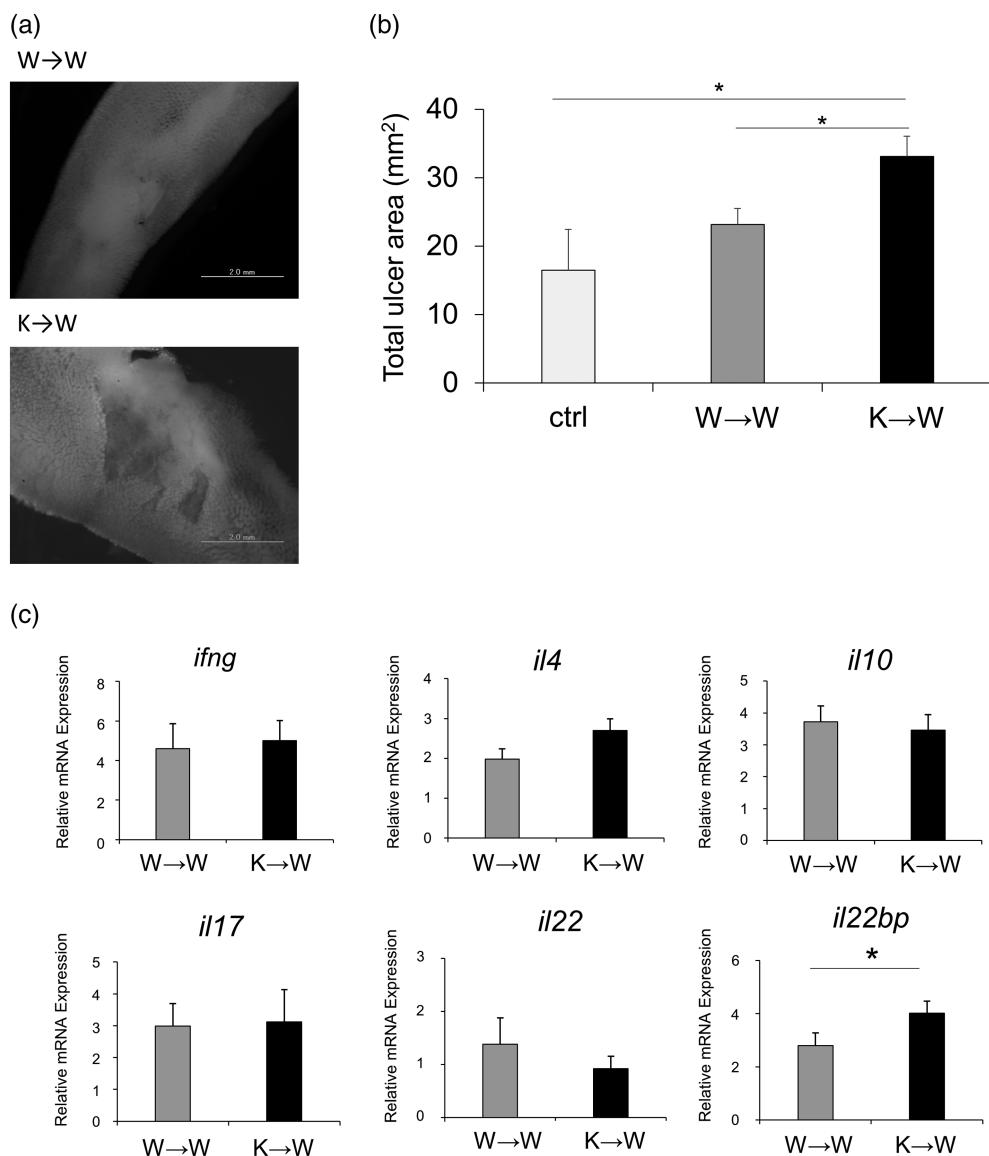


Figure 6 Adoptive transfer of CD103⁺ DCs from WT or *Ccr7*^{-/-} mice. Representative pictures of ulcers in the small intestine after transferring CD11c⁺ CD103⁺ DCs derived from WT or *Ccr7*^{-/-} mice (a). Summations of ulcerated areas in the SI of WT mice (W) administered indomethacin after transferring of CD103⁺ DCs derived from WT (W) or *Ccr7*^{-/-} mice (K). Control (Ctrl) represents WT mice without the transfer of CD103⁺ DCs. Data are expressed as the mean + standard error of four mice per group. There was a significant difference in the distribution among the three groups by ANOVA ($P = 0.026$) and the difference between the groups were analyzed by Student's *t*-test. * $P < 0.05$ (b). Expression of cytokine mRNA in the mononuclear cells of SI-LP was analyzed by qRT-PCR after adoptive transfer of CD103⁺ DCs isolated from WT (W → W) or *Ccr7*^{-/-} mice (K → W) (c). Data are expressed as the mean + standard error of three to six mice per group. * $P < 0.05$ by Student's *t*-test.

inflammation, whereas mice received *Il22bp*^{-/-} T cells were largely protected from disease development.³⁷ In our experiments, IL-22BP expression in *Ccr7*^{-/-} mice was mainly observed CD103⁺ DCs at significantly higher levels compared with WT mice (Fig. 4a). In addition, our data clearly demonstrated that transfer of CD103⁺ DCs isolated from *Ccr7*^{-/-} mice induced more severe intestinal inflammation than in CD103⁺ DCs isolated from WT mice. Thus, our study clearly revealed the importance of CD103⁺ DCs in the control mucosal homeostasis through the regulation of IL-22BP. Recruitment of lymphocytes controlled by CCR7 is also affected by the cell adhesion molecules and

selectins, such as MAdCAM-1 and L-selectin.^{40,41} Alteration of CCR7 expression may change the signaling pathways related to these adhesion molecules and selectins and these molecular changes may affect cellular signaling pathways. These mechanisms need to be clarified in the future study.

Our data showed that the expression of REG1, a downstream molecule of IL-22, was decreased in the SI epithelial cells of *Ccr7*^{-/-} mice. In addition, *CCR7*^{-/-} mice administered with indomethacin showed extensive alteration of the fecal microbiota with decreased diversity of bacterial flora. REG family proteins are involved in cellular proliferation in gastrointestinal cells.^{42,43}

Among the REG family members, NSAID-induced enteritis is reported to be exacerbated in *Reg1*-deficient mice and the administration of recombinant REG1 can suppress enteritis.⁴⁴ IL-22 and antimicrobial products are also known to influence commensal microbe composition in the intestine and, consequently, the efficiency of pathogen clearance.⁴⁵ We observed the alterations in fecal microbiota in *Ccr7*^{-/-} mice, but it was not yet clarified whether IL-22BP or REG1 is responsible for the alteration of fecal microbiota and the underlying mechanism should be clarified in the future study.

In conclusion, our study has identified importance of IL-22BP in association with CCR7 and CD103⁺ DCs in the pathogenesis of NSAID-induced enteropathy and IL-22BP may be a potential therapeutic target for NSAID-induced enteropathy.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Confirmation of CCR7 deficiency by flow cytometry. Splenic mononuclear cells were stained with antibodies specific for CD4 and CCR7. Representative histograms for the expression of CCR7 in CD4 $^{+}$ cells are shown.

Figure S2. Expression of *Il1b* and *Il6* mRNA in the mononuclear cells of SI-LP of WT and *Ccr7* $^{-/-}$ mice administered indomethacin. The mRNA expression of *Il1b* (A) and *Il6* (B) in WT (■) and *Ccr7* $^{-/-}$ mice (■) is shown. Data are expressed as the mean + SE. * $P < 0.05$ by Student's t-test of three mice per group.

Figure S3. Expression of prostaglandin E2 in the small intestine of WT and *Ccr7* $^{-/-}$ mice administered indomethacin. The levels of prostaglandin E2 produced in the SI of in WT (■) and *Ccr7* $^{-/-}$ (■) mice were measured by enzyme-linked immunosorbent assay and were not significantly different between the two groups. The data are expressed as the mean + standard error of three mice per group.

Figure S4. Metagenomic analysis of fecal samples obtained from WT or *Ccr7* $^{-/-}$ mice with or without indomethacin administration. Alpha-(A) and beta-(B) diversity of fecal microbiota of WT and *Ccr7* $^{-/-}$ mice with or without indomethacin administration. The Shannon index was used to express alpha diversity. Fecal samples were further analyzed to determine the proportions of the phylum levels (C). IND, Indomethacin. Data were obtained from three mice per group.

Metagenomic analysis