Interleukin-33 delays recovery of mucosal inflammation via downregulation of homeostatic ABCG5/8 in the colon

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Abstract

Previous studies have suggested that interleukin-33 (IL-33) is involved in the pathogenesis of ulcerative colitis (UC), though the detailed mechanisms are not fully known. We investigated IL-33-mediated colonic homeostasis using a mechanistic method. *Il33*^{−/−} mice were more tolerant to dextran sulfate sodium-induced acute colitis than the wild type and also showed delayed recovery from colitis with recombinant IL-33 (rIL-33) administration. Unexpectedly, microarray analysis identified significant downregulation of the *Abcg5/8* genes in mouse colons following rIL-33 treatment. ABCG5/8 are known cholesterol transporters in the small intestine and liver, though their colon activities have not been elucidated, thus their role in IL-33-mediated inflammation was investigated. In vitro, toll-like receptor (TLR) stimulation upregulated ABCG5/8 mRNA expression in Caco2 and HCT-15 cells, with subsequent downregulation by rIL-33, while inhibition of ABCG5/8 along with their siRNA increased TLR-stimulated IL-8 production. Together, these results indicated that colonic ABCG5/8 play a regulatory role in TLR-induced inflammation, while histological inflammation in human UC was correlated positively with the level of mucosal IL-33 and inversely with that of colonic ABCG5/8. This is the first report of IL-33-mediated downregulation of colonic ABCG5/8 in a colitis recovery phase, indicating their involvement in UC pathogenesis and potential as a therapeutic target.

Introduction

Inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis (UC), are chronic intestinal immune disorders characterized by inadequate immune response to enteric resident bacteria in susceptible hosts [1–3]. Despite numerous basic and clinical IBD research projects, the pathogenesis of IBD remains elusive, and current treatment strategies are mainly reliant on anti-inflammatory or immune suppressive drugs rather than

radical treatment. Therefore, new mechanistic studies to unveil the pathogenesis of IBD are urgently anticipated.

Interleukin-33 (IL-33), a member of the IL-1 family, drives T helper-2 (Th2) immune responses via ligation to the ST2 receptor [4]. It is produced by a variety of cell types, including fibroblasts, mast cells, dendritic cells, macrophages, osteoblasts, endothelial cells, and epithelial cells, while ST2 is highly expressed in Th2 cells, mast cells, and group 2 innate lymphocytes [5]. IL-33 and ST2 are likely involved in the pathogenesis of IBD [6, 7], and IL-33 in intestinal mucosa is elevated in active IBD patients [7, 8], while genetic polymorphisms of the IL-33 pathway are related to increased risk for IBD in humans [9]. However, controversial results in regard to the biological effects of IL-33 in animal models of experimental colitis have been presented. Some studies have indicated that IL-33 has a pro-inflammatory role in colitis [10–14], while others have suggested a protective role [15–18]. Therefore, additional mechanistic studies to reveal IL-33-mediated mucosal inflammation are required for a better understanding of the pathogenesis of IBD, including that in UC patients.

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Adenosine triphosphate (ATP)-binding cassette subfamily G members 5 and 8 (ABCG5/8) function as heterodimers, and play important roles in lipid metabolism as cholesterol or sterol transporters in the small intestine and liver [19–21]. Genetic ablation of ABCG5/8 increases the fractional absorption of dietary plant sterols and cholesterol, resulting in hypercholesterolemia or hypersitosterolemia [22]. As a result, most studies that have investigated ABCG5/8 focused on the small intestine or liver, while the roles of these transporters in colon and colonic disorders are entirely unknown.

In the present study, we examined possible mechanisms related to IL-33 sustaining mucosal inflammation via downregulation of homeostatic ABCG5/8 in the colon. Our findings may help to understand the IL-33-mediated pathogenesis of IBD, especially in patients with UC, and potentially contribute to create a new treatment strategy for IBD.

Materials and methods

Mice

BALB/c wild-type (WT) mice were purchased from CLEA Japan (Tokyo, Japan). After an initial adaptation period of 2–4 week in specific pathogen-free (SPF) conditions at Shimane University, those mice were used for the experiments at 8–12 weeks of age. *Il33*^{−/−} mice (acc. no. CDB0631K; www2.clst.riken.jp/arg/mutant%20mice%20list.html) were originally obtained from RIKEN Center for Developmental Biology (Kobe, Japan) [10], then maintained in SPF conditions (in the same room as WT mice). The offspring were used for the experiments at 8–12 weeks of age. All mice were housed in the same animal room.

Colon biopsies in UC patients and healthy volunteers

Eleven patients previously diagnosed with UC and who underwent a colonoscopy were enrolled (Table 3). Biopsy specimens were taken from an endoscopically active (Mayo score > 1) and/or inactive part (Mayo score 0 or 1) of the colon of each patient (Table 3). As a control, distal colon samples obtained from healthy volunteers were also assessed. Patients who had colon cancer or an artificial anus, or were regularly taking aspirin and/or other non-steroidal anti-inflammatory drugs were excluded. One of the samples obtained from each patient and volunteer was processed for real-time PCR analysis, while the other was formalin fixed and embedded in paraffin for histological assessment.

Dextran sulfate sodium (DSS)-induced colitis model

The present mice had a BALB/c background and exhibited distal-dominant colitis as a DSS-induced colitis model [23]. A DSS solution (5 kDa; Wako Pure Chemical Industries, Osaka, Japan) (3% w/v) was administered as drinking water for 7 or 10 days to induce colitis, with the solution changed every other day, as previously described [24]. Mouse conditions were carefully monitored and weights recorded three times a week after beginning DSS administration until termination of the experiment. The percent body weight gain as compared with the initial weight was calculated. Each mouse was euthanized with carbon dioxide (CO₂), then the distal colon was collected for histology and RNA examinations.

Histological examinations

Histological samples from mice were fixed in 10% formalin, then sections were prepared and stained with hematoxylin and eosin (HE) to visualize the general morphology. Scoring for colitis in the distal colon was performed in a blinded manner, as previously described [25], with some modifications. Briefly, severity of inflammation (based on polymorph nuclear neutrophil infiltration; 0–3: none, slight, moderate, severe), depth of injury (0–3: none, mucosal, mucosal and submucosal, transmural), and crypt damage (0–4: none, basal one-third damaged, basal two-thirds damaged, only surface epithelium intact, entire crypt and epithelium lost) were the parameters considered. The score for each was added, with a maximum sum value of 10.

Each histological section from the human samples was stained with HE, then assigned an inflammatory activity score using the Matts histological grading system as follows: grade 1, completely normal appearance; grade 2, some infiltration of the mucosa or lamina propria with either round cells or polymorphonuclear leukocytes; grade 3, marked cellular infiltration of all layers of the mucosa; grade 4, presence of crypt abscesses, with marked infiltration of all layers of the mucosa; and grade 5, ulceration, erosion, or necrosis of the mucosa, with cellular infiltration in some or all layers.

Immunohistochemistry

Distal colon, terminal ileum, liver, and spleen samples from WT and *Il33*^{−/−} mice, and colonic biopsy specimens obtained from UC patients and healthy volunteers were used. Samples were fixed in 10% formalin and paraffin sections were prepared for immunohistochemistry. After deparaffinization, endogenous peroxidase activity was blocked with Peroxidase-Blocking Solution (Dako) for

10 min, then sections (5 µm thick) were washed with PBS, and incubated with anti-human/mouse ABCG5 and eight antibodies (Santa Cruz Biotechnology) at room temperature for 30 min. After washing again with PBS, the bound antigen–antibody complex was detected using a Chem-Mate™ DAKO EnVision™ Detection Kit (DAKO).

Cell cultures

Caco2 and HCT-15 human colon cancer cell lines were cultured in 24-well plates for 24 h at 37 °C with 5% CO₂, then used when reaching 60–80% confluence. The culture medium was RPMI 1640 (Gibco-Invitrogen, Carlsbad, CA), with 10% fetal bovine serum (FBS; Gibco-Invitrogen) added for Caco2 or 20% FBS for HCT-15 cells, and 1% penicillin-streptomycin-amphotericin B (Gibco-Invitrogen) with or without the following bacterial products: 1 µg/ml of ultra-pure *E. coli* lipopolysaccharide (LPS) (0111:B4 strain) (Invivogen, San Diego, CA) and 100 ng/ml of flagellin (Sigma-Aldrich). Different doses of human recombinant IL-33 (rIL-33) (R&D systems Minneapolis, MN) were selectively added. Following the cell cultures, supernatants were collected for measuring cytokines by ELISA, while the cells were analyzed for gene expression. In some of the experiments, siRNA for *Abcg5*, *Abcg8*, or both *Abcg5* and *8*, or scramble RNA was added prior to beginning the cell culture. All siRNAs and scramble RNA products were purchased from Santa Cruz, and used according to the manufacturer's protocol.

Cytokine measurements

IL-8 concentrations were measured by ELISA using a human anti-IL-8 ELISA kit (Invitrogen). Cytokine concentrations in culture supernatants were determined in triplicate and compared with standard curves generated using the appropriate recombinant cytokine, according to the manufacturer's instructions.

Real-time polymerase chain reaction assays

Total RNA was isolated from distal colon specimens using an RNeasy Mini Kit (Qiagen Sciences, Valencia, CA). First-strand complementary DNA (cDNA) was synthesized from 1 µg of total RNA using M-MLV Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions. Quantitative reverse-transcription polymerase chain reaction assays were performed with a Mastercycler EP realplex 2S (Eppendorf, Wesseling-Berzdorf, Germany) using SYBR green quantitative PCR SuperMix (Invitrogen) to quantify gene expression. The following PCR primers were used in this study. Human primers: hAbcg5-F TGG ACCAGGCAGATCCTCAAA and hAbcg5-R CCGTTCA

CATACACCTCCCC; hAbcg8-F AGCCTCCTTGCTAGA TGTGAT and hAbcg8-R GTCTCTCGCACAGTCAAG TTG; hST2-F GACGGCGACCAGGTCCTT and hST2-R GGGCTCCGATTACTGGAAACA; hGapdh-F ACAACT TTGGTATCGTGGAAGG and hGapdh-R GCCATCAC GCCACAGTTTC; hIL33-F CACCCCTCAAATGAATCA GG and hIL33-R GGAGCTCCACAGAGTGTTC; hCldn1-F CCTCCTGGGAGTGATAGCAAT and hCldn1-R GGCAACTAAAATAGCCAGACCT; hOcIn-F: ACAAGC GGTTTTATCCAGAGTC and hOcIn-R GTCATCCACA GGCGAAGTTAAT; hIl1b-F TTCGACACATGGGATA ACGAGG and hIl1b-R TTTTGTGCTGTGAGTCCCGGAG. Mouse primers: mIL6-F CTGCAAGAGACTTCCATC CAG and mIL6-R AGTGGTATAGACAGGTCTGTTGG; mTnfα-F ACCCTCACACTCAGATCATCTTCTC and mTnfα-R TGAGATCCATGCCGTTGG; mAbcg5-F TCCTG CATGTGTCCTACAGC and mAbcg5-R ATTTGCCTG TCCCACTTCTG; mAbcg8-F AACCCTGCGGACTTC TACG and mAbcg8-R CTGCAAGAGACTGTGCCTTCT; mIl1b-F GAAATGCCACCTTTTGACAGTG and mIl1b-R TGGATGCTCTCATCAGGACAG; and mGapdh-F AGGT CCGTGTGAACGGATTTG and mGapdh-R TG TAGAC CATGTAGTTGAGGTCA. Each cDNA sample was analyzed in duplicate for quantitative assessment of RNA amplification and the results are expressed as relative to the housekeeping gene *Gapdh*. The $\Delta\Delta C_t$ method was used to assess relative quantifications.

Microarray analysis

Total RNA was prepared from distal colon samples obtained from DSS-treated *Il33*^{−/−} mice treated with 1 µg of rIL-33 or PBS, as described above. RNA samples were sent to Filgen (Aichi, Japan), where DNA microarray analyses were performed. Briefly, 500 ng of RNA was reverse transcribed into cDNA and transcribed into cRNA. Amplified sense-strand cDNAs were synthesized with cRNA, then fragmented and labeled using a Gene-Chip WT PLUS Reagent Kit (Thermo Fisher Scientific, Kanagawa, Japan). Next, the samples were hybridized to an Affymetrix GeneChip Mouse Gene 2.0 ST whole transcript-based array (Thermo Fisher Scientific), according to the manufacturer's protocol. The hybridized arrays were washed and scanned with a Gene chip Scanner 30007G (Thermo Fisher Scientific). All data were transferred to the Affymetrix Expression Console Software package, ver. 4.0 (Thermo Fisher Scientific) and normalized using the robust multiarray average method, then gene expression levels were obtained. Fold changes and *p* values were used to identify differentially expressed genes (DEGs). The threshold was set at a fold change ≥ 2.0 or <0.5 , and a *p* value ≤ 0.05 . To fully clarify the biological meaning of the DEGs, a gene ontology (GO)

Table 1 Microarray analysis

Upregulated genes	<i>p</i> value	Gene description
<i>Serpina3n</i>	4.67E−05	Serine (or cysteine) peptidase inhibitor, clade A, member 3 N
<i>Mcpt1</i>	4.67E−05	Mast cell protease 1
<i>Apod</i>	4.67E−05	Apolipoprotein D
<i>Reg4</i>	4.67E−05	Regenerating islet-derived family, member 4
<i>Pla2g2a</i>	4.67E−05	Phospholipase A2, group IIA (platelets, synovial fluid)
<i>Fos</i>	0.00232	FBJ osteosarcoma oncogene
<i>Cyp4f14</i>	0.00558	Cytochrome P450, family 4, subfamily f, polypeptide 14
<i>Cpa3</i>	0.04352	Carboxypeptidase A3, mast cell
Downregulated genes	<i>p</i> value	Gene description
<i>Cpm</i>	0.000712	Carboxypeptidase M
<i>Slc46a1</i>	0.000712	Solute carrier family 46, member 1
<i>Ighm</i>	0.000712	Immunoglobulin heavy constant mu
<i>Glycam1</i>	0.000712	Glycosylation dependent cell adhesion molecule 1
<i>Iglv1</i>	0.000712	Immunoglobulin lambda variable 1
<i>Cldn1</i>	0.000712	Claudin 1
<i>Sfrp2</i>	0.000712	Secreted frizzled-related protein 2
<i>Steap4</i>	0.000712	STEAP family member 4
<i>Scnn1g</i>	0.000712	Sodium channel, nonvoltage-gated 1 gamma
<i>Scnn1b</i>	0.000712	Sodium channel, nonvoltage-gated 1 beta
<i>Olfir78</i>	0.000712	Olfactory receptor 78
<i>Plet1</i>	0.000712	Placenta expressed transcript 1
<i>P2ry4</i>	0.000712	Pyrimidinergic receptor P2Y, G-protein coupled, 4
<i>Edn1</i>	0.01835	Endothelin 1
<i>Igj</i>	0.01835	Immunoglobulin joining chain
<i>Abcg8</i>	0.01866	ATP-binding cassette, subfamily G (WHITE), member 8
<i>Abcg5</i>	0.01866	ATP-binding cassette, subfamily G (WHITE), member 5
<i>Casp14</i>	0.0286	Caspase 14
<i>Nr1d1</i>	0.0286	Nuclear receptor subfamily 1, group D, member 1
<i>Klk15</i>	0.03863	Kallikrein related peptidase 15

Mouse Gene ST (Filgen, Aichi, Japan) assays were performed with colonic samples from DSS-treated *Il33*^{−/−} mice injected with 1 µg rIL-33 or PBS (related to Fig. 2). Up- and downregulated genes with a *p* value less than 0.05 are listed

classification system and NCBI BioSystems (<https://www.ncbi.nlm.nih.gov/biosystems>) were used with a Microarray Data Analysis Tool (ver. 3.2, Filgen) (Tables 1 and 2).

Statistical analysis

Using the Prism 6 software package (GraphPad, San Diego, CA, USA), a Mann–Whitney U test or unpaired *t* test was used to compare mean values between two groups, while comparisons of mean values for multiple groups were analyzed with one-way analysis of variance and Tukey's post hoc test. For microarray findings, *p* values for GO analysis and pathway analysis were calculated using a two-tailed Fisher's exact test with the

manufacturer's software (Filgen). *p* values less than 0.05 were considered to indicate significance.

Ethical statement

The study protocol of the mouse study was approved by the Institute for Animal Experimentation of Shimane University (protocol no. IZ28-38), and the experiments were performed in accordance with the guidelines for the care and use of laboratory animals established by the National Institutes of Health. The protocol for the human study was approved by the ethical committee of Shimane Hospital (study no. 2778) and performed in adherence to the Helsinki Declaration. Each patient and healthy volunteer provided informed written consent prior to participation.

Table 2 Pathway analysis

Upregulated pathway	<i>p</i> value
Arachidonic acid metabolism	0.004
Renin-angiotensin system	0.019
alpha-linolenic acid metabolism	0.025
MAPK signaling pathway	0.025
Phospholipases	0.035
Pathways in cancer	0.038
Ether lipid metabolism	0.038
Fat digestion and absorption	0.040
Linoleic acid metabolism	0.044
Downregulated pathway	<i>p</i> value
ABC transporters	0.003
Fat digestion and absorption	0.003
Aldosterone-regulated sodium reabsorption	0.003
Bile secretion	0.008
Cell adhesion molecules (CAMs)	0.030
Vitamin digestion and absorption	0.045

Based on records in a gene association database, pathway analysis of microarray analysis findings was additionally performed (Table 1, related to Fig. 2)

Results

Il33^{−/−} mice tolerant to acute DSS-induced colitis

Previous studies have presented conflicting results in terms of the effect of IL-33 on colitis, thus we initially investigated the influence of IL-33 using a DSS-induced acute colitis model, in which distal-dominant colitis resembling human UC develops, by comparing *Il33*^{−/−} mice with BALB/c WT mice. The *Il33*^{−/−} mice developed less severe colitis as compared with the WT mice, which was shown by their tolerance to body weight loss (Fig. 1a) and less severe histological scores (Fig. 1b, c), while mucosal pro-inflammatory *Tnfa*, *Il6*, and *Il1b* levels were more pronounced in the WT mice as compared with the *Il33*^{−/−} mice (Fig. 1d). These results indicate that IL-33 exaggerates DSS-induced colitis in the induction phase.

rIL-33 treatment delays recovery from acute colitis in *Il33*^{−/−} mice

It was previously demonstrated that IL-33 has a protective role in chronic colitis by upregulating regulatory T and B cells [17, 18], thus we initially speculated that rIL-33 treatment would induce prompt amelioration in the recovery phase of DSS-treated *Il33*^{−/−} mice. Based on prior investigations, we used two different doses of rIL-33, 1 µg and 0.1 µg/mouse [12, 16, 17, 26]. Interestingly, rIL-33 delayed

recovery from body weight loss (Fig. 2a) and sustained mucosal inflammation in a dose-dependent manner (Fig. 2b–d), suggesting that it plays a pathogenic role in the recovery phase of acute colitis. In addition, our findings indicate that IL-33 may be an important factor driving development of acute colitis in cases of chronic intestinal inflammation.

IL-33 downregulates colonic *Abcg5* and 8 in recovery phase of colitis

Next, we sought to determine the mechanisms underlying IL-33-mediated sustained inflammation in the recovery phase of DSS-induced colitis. To clarify this issue, we performed microarray analysis of colon samples obtained from *Il33*^{−/−} mice treated with PBS or 1 µg of rIL-33 (R&D Systems, Minneapolis, MN), the latter of which resulted in up- and downregulation of multiple genes (Table 1). Additional pathway analysis indicated that multiple pathways, including arachidonic acid metabolism and the MAPK pathway, were upregulated by rIL-33 treatment, while some biological pathways were markedly downregulated (Table 2). Although most of the identified pathways were anticipated based on previous findings, downregulation of the ABC transporter G5 and G8 genes (*Abcg5/8*) as well as their related pathways was a totally unexpected result, because the involvement of *Abcg5/8* in colon diseases including IBD has not been previously reported.

Next, we examined the distribution of *Abcg5/8* in WT mice using real-time PCR and immunohistochemistry. *Abcg5/8* expressions were clearly seen in the distal colon as well as the small intestine, while the levels in the liver were much greater (Supplementary Fig. 1A). Immunohistochemistry findings showed that ABCG5 was located in the cytoplasm of epithelium cells of colons obtained from the WT mice without colitis, while colonic ABCG8 was predominantly seen on the epithelium surface (Supplementary Fig. 1B). The *Il33*^{−/−} mice also showed higher levels of *Abcg5/8* gene expression and ABCG5/8 staining in both the small intestine and distal colon as compared with the WT mice (Supplementary Fig. 2A, B), suggesting that IL-33 negatively regulates *Abcg5/8* expression. In addition, rIL-33 significantly downregulated the expressions of *Abcg5/8* in a dose-dependent manner in the colons of DSS-treated *Il33*^{−/−} mice (Fig. 3a). These observations were confirmed by immunohistochemistry findings, which showed that DSS-treated colons of *Il33*^{−/−} mice had decreased levels of ABCG5/8 expression as compared with the PBS-treated colons of those mice (Fig. 3b). We also noticed that ABCG8 was stained in the cytoplasm of epithelium cells in the mice with DSS treatment, indicating that inflammation may regulate the expression level and site of ABCG8 (Fig. 3b,

Fig. 1 *Il33*^{-/-} mice tolerant to acute DSS colitis. **A** A 3% DSS solution was continuously administered to 8-week-old WT and *Il33*^{-/-} mice for 10 days, after which they were euthanized and the colons were obtained for evaluating colitis. **a** Body weight changes. **b, c** Histological colitis scores and representative histological images of distal colon samples (×40 magnification). **d** Gene expressions in distal colon samples were evaluated by real-time PCR and normalized using mRNA levels in WT mice. *N* = 9 mice/group, combined from two independent experiments. Values are presented as the mean ± SE. *p < 0.05, **p < 0.01. Mann–Whitney U test

Supplementary Fig. 2B). In addition, *Abcg5/8* expressions in the colon from WT mice treated with rIL-33 in the recovery phase of DSS-induced colitis were examined following administration of rIL-33, which showed a delay in recovery from DSS-induced colitis with a massive reduction of *Abcg5/8* levels (Supplementary Fig. 3A–C). Together, these results suggest that IL-33 sustains inflammation via downregulation of *Abcg5/8* in the colon.

IL-33 downregulates toll-like receptor (TLR)-activated upregulation of *ABCG5/8* in colon cell lines

Since IL-33 was found to sustain colonic inflammation and downregulate *ABCG5/8* in the colon, we next sought to determine the effects of IL-33 on *ABCG5/8* colonic expression in vitro using human colon cancer cell lines. We stimulated Caco2 and HCT-15 cells with a TLR 4 ligand LPS or TLR5 ligand flagellin along with different doses of rIL-33, and analyzed *ABCG5/8* gene expression and IL-8 production. Both LPS and flagellin upregulated *ABCG5/8* mRNA expression levels in the absence of rIL-33, while addition of IL-33 decreased those (Fig. 4a, b). These findings indicate that *ABCG5/8* gene expression in the colon is tightly regulated by TLR stimulation and IL-33. In addition, rIL-33 was found to increase IL-8 production in the presence but not absence of TLR stimulation (Fig. 4c), suggesting that IL-33 has a pro-inflammatory role only when TLR stimulation is present. Taken together, *ABCG5/8* may

have anti-inflammatory roles in TLR-mediated colonic inflammation that are negated by IL-33.

ABCG5/8 protective against TLR-mediated colonic inflammation

Next, we examined the roles of colonic *ABCG5/8* against inflammation based on our speculation that its downregulation would exaggerate TLR-mediated inflammation. For these experiments, we performed knockdown of the *ABCG5/8* genes in Caco2 and HCT-15 cells by siRNA, and determined pro-inflammatory IL-8 production after stimulation with TLR ligands. Interestingly, blockade of the *ABCG5/8* genes resulted in pronounced TLR-stimulated IL-8 production by the colon cell lines (Fig. 5a, b). These findings indicate that TLR-activated colonic *ABCG5/8* have important protective roles for maintaining gut homeostasis, though their expression is subsequently suppressed when IL-33 is present.

Since microarray results also showed significant downregulation of tight junction-related genes and their pathways (Tables 1 and 2), we investigated the involvement of such genes in this system. *ABCG5/8* siRNAs significantly decreased the mRNA expressions of *CLAUDIN-1* (*CLDN1*) and *OCCLUDIN* (*OCLN*), and simultaneously upregulated that of pro-inflammatory *IL1B* (Fig. 5c). In addition, in colons obtained from *Il33*^{-/-} mice treated with rIL-33, we noted significant downregulation of the tight junction genes

Fig. 2 Recombinant IL-33 treatment delays recovery of colitis in *IL33*^{-/-} mice. **a** A 3% DSS solution was administered to 12-week-old *IL33*^{-/-} mice for 7 days. After DSS treatment, PBS, 0.1 µg of recombinant IL-33 (rIL-33), or 1 µg of rIL-33 was injected *i.p.* daily, as indicated in the figure. After 14 days, the mice were euthanized and the colons were obtained for evaluating colitis. **b** Body weight changes. **c** Histological

scores. **d** Representative histological images of distal colon samples (×40 magnification). **e** mRNA expression in the distal colon was determined using real-time PCR and normalized to that of PBS-treated mice. $N = 7-9$ mice/group, combined from two independent experiments. Values are shown as the mean ± SE. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ANOVA with Tukey's post hoc test

Ocln, Cldn1, and Zo1 as compared with those from mice treated with PBS (Supplementary Fig. 4).

Collectively, our findings indicate that IL-33 sustains mucosal inflammation via *ABCG5/8* downregulation, which might lead to dysfunction of tight junction-related genes, indicating a key mechanism of IL-33-dependent exacerbation of colitis. Additional investigations of the role of colonic *ABCG5/8* in maintaining tight junction proteins will be required in a future study.

Colonic *ABCG5/8* downregulated in human UC patients

Based on our findings that TLR-stimulated *Abcg5/8* expressions are downregulated in the presence of IL-33 in mice, we sought to verify the association of colonic *ABCG5/8* with IL-33 as well as inflammation in human cases of UC (Table 3). We obtained biopsy samples from both endoscopically active and inactive areas of colons of UC patients, confirmed by histological findings, as well as samples from healthy subjects and examined them

(Table 3). In the specimens from inflamed areas of the UC patients, it was notable that colonic *ABCG5/8* gene expression was significantly decreased, while *IL-33* and *IL1B* were upregulated as compared with specimens from inactive areas in UC patients as well as from healthy subjects (Fig. 6a). Furthermore, *IL-33* and *IL1B* gene expressions in the active areas showed greater upregulation as compared with inactive areas in the same subject (Supplementary Fig. 5A). On the other hand, colonic *ST2* gene expression did not differ among any of the obtained specimens (Supplementary Fig. 5B). Immunohistochemistry results showed faint levels of *ABCG8* staining in samples from the active colon parts of UC patients, as compared with the inactive colon parts and healthy subject samples (Fig. 6b). In addition, the presence of *IL-33* was shown to be related to scoring for severity of histological colitis, while that of *ABCG5* was inversely correlated with histological inflammation (Supplementary Fig. 6). These findings confirmed our results obtained in the mouse experiments and highlight the importance of IL-33-mediated *ABCG5/8* regulation in human UC. Taken

Fig. 3 IL-33 treatment decreases expression of *Abcg5* and 8 genes. *Il33*^{-/-} mice were treated with DSS and injected with PBS, 0.1 µg of rIL-33, or 1 µg of rIL-33, then the distal colons were obtained on day 13 analyzed (related to Fig. 2). **a** *Abcg5* and *Abcg8* gene expressions in the distal colon were determined using real-time PCR, then normalized to those seen in PBS-treated mice. *N* = 7–9 mice/group. Values are

shown as the mean ± SE. *p < 0.05, **p < 0.01. ANOVA with Tukey's post hoc test. Expression site and degree of ABCG5 and ABCG8 in colons of *Il33*^{-/-} mice treated PBS or 1 µg of rIL-33 were confirmed using immunohistochemistry. Representative images are shown in **b**. ×40 and ×100 magnification

together, the present study identified homeostatic roles of colonic ABCG5/8 underlying IL-33-mediated mucosal injury in colon specimens obtained from both mice and humans.

Discussion

The present study was conducted to address the possible mechanisms underlying IL-33-mediated mucosal inflammation in colon tissues. An unexpected observation was the involvement of colonic ABCG5/8, previously reported to function as cholesterol transporters in the small intestine and liver. Our novel findings may help to elucidate the molecular mechanisms involved in progression from acute colitis to chronic mucosal inflammation that occurs in UC patients.

In allergic diseases, including asthma, atopic dermatitis, and eosinophilic intestinal disorders, IL-33 has a pathogenic role, as it worsens Th2-immune responses and activates eosinophils or mast cells, indicating it to be a possible ideal

treatment target. On the other hand, IL-33 has various effects in terms of IBD, as it has been shown to play a pro-inflammatory role in experimental colitis through induction of Th2-dominant immune response [10–14], whereas others have reported that IL-33 can induce regulatory T cells and B cells that protect from exacerbation of colitis [15–18]. These contrasting results may be related to the timing and/or immunological features of colitis, including mouse background, or the quality and/or quantity of the targeted immune cells. In the present study, IL-33 was shown to sustain mucosal inflammation in the recovery phase of acute DSS colitis. DSS induces both Th1 and Th2-immune reactions, which are dependent on timing (acute or chronic phase) or animal background [27]. Thus, the recovery phase of DSS colitis tends to be Th2-dominant and might be exaggerated by IL-33, as demonstrated by the present results. In addition, we used mice with a BALB/c background, which are susceptible to Th2-mediated immune reactions. Although IL-33 ameliorates chronic colitis by inducing regulatory T and B cells [17, 18], its effects may not be adequately long lasting for adequate numbers of

Fig. 4 TLR-induced *ABCG5/8* expression downregulated by rIL-33. **a** Caco2 cells were cultured in 12-well-plates with or without 1 μ g/ml of LPS in the presence or absence of different doses of rIL-33 (0, 10, 100, and 1000 ng/ml). **b** HCT-15 cells were cultured in 12-well-plates with or without 100 ng/ml of flagellin in the presence or absence of different doses of rIL-33 (0, 10, 100, and 1000 ng/ml). After 6 h, cells were

harvested and expression levels of *ABCG5/8* were determined by real-time PCR. **c** Levels of IL-8 in supernatant from cultures noted in **b** were measured by ELISA. $N=4$ /group, combined from two independent experiments. Values are shown as the mean $\pm$ SE. * $p < 0.05$, ** $p < 0.01$. Mann–Whitney U test and ANOVA with Tukey's post hoc test

Fig. 5 *ABCG5/8* siRNA increases TLR-stimulated IL-8 production. **a** Caco2 or **b** HCT-15 cells were incubated with siRNA for *ABCG5*, *ABCG8*, *ABCG5/8*, or scramble RNA for 6 h, then stimulated for 24 h with (a) 1 μ g/ml of LPS or (b) 100 ng/ml of flagellin. Culture supernatants were assessed for IL-8 by ELISA. $N=6$, combined from two independent experiments. Values are shown as the mean $\pm$ SE. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ANOVA with Tukey's post hoc test. **c** Gene expressions of CLAUDIN-1 (*CLDN1*), OCLUDIN (*OCLN*), and *IL1B* in flagellin-stimulated HCT-15 cells treated with scramble RNA or siRNAs for *ABCG5/8* (related to **b**) were determined by real-time PCR. * $p < 0.05$, Mann–Whitney U test

Table 3 Clinical characteristics and the biopsy samples of UC patients

Clinical characteristics		Biopsy samples from UC patients		
Patient number	11	Endoscopic finding	Noninflamed	Inflamed
Age, years	47.7 (25–66)	Sample number	10	11
Sex (male: female)	10:2	Location		
Disease extent		Cecum	2	0
Pancolitis	6	Ascending colon	3	1
Left-sided colitis	5	Transvers colon	2	0
Proctitis	1	Descending colon	1	2
Disease severity		Sigmoid colon	1	2
Mild	2	Rectum	1	6
Moderate	6	Mayo score		
Severe	3	0	10	0
Treatment		1	0	2
5-ASA	9	2	0	7
Steroid	4	3	0	2
Anti-TNF	0			
Tacrolimus	4			
Azathioprine	2			

We enrolled 11 UC patients previously diagnosed with UC. Colonic biopsy specimens were obtained from endoscopically active (inflamed) and inactive (noninflamed) areas of UC patients. One patient with pancolitis did not possess an inactive area, thus we analyzed samples from 11 active and 10 inactive areas. Endoscopic findings regarding the degree of inflammation were confirmed by histology results

Fig. 6 IL-33 elevated in active UC patients in parallel with ABCG5/8 downregulation. Biopsy samples were obtained from endoscopically active ($N=11$) and inactive ($N=10$) parts of the colon of UC patients, as well as healthy volunteers (HV) ($N=5$). Gene expressions were determined using real-time PCR and compared with those in

samples from the control group. **a** *IL-33*, *IL1B*, and *ABCG5/8* gene expressions were determined by real-time PCR, and normalized to that of *GAPDH*. **b** Immunohistochemistry for ABCG8 (×100 magnification). Values are shown as the mean ± SE. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. ANOVA with Tukey's post hoc test

regulatory cells to be induced in the present DSS model. These issues should be further addressed using another experimental colitis model with a different background in a future study.

Another possibility is that IL-33 impairs barrier functions in colonic epithelium cells via downregulation of tight junction proteins. Dysfunction of intestinal tight junctions results in invasion by pathogenic microorganisms, and initiation and sustaining of mucosal inflammation [20, 28], while others have demonstrated that IL-33 in cooperation with IL-4 causes dysfunction of airway epithelium and endothelial cells [29, 30]. The present microarray analysis showed that the tight junction pathway is significantly downregulated by rIL-33 treatment, which is consistent with the observations noted above and might be associated with delay in recovery from mucosal inflammation.

The most interesting finding obtained in this study is dysfunction of colonic ABCG5/8 induced by IL-33 in cases of sustained mucosal inflammation. Although we and others have confirmed that ABCG5/8 are present in the colon (Supplementary Fig. 1A and B) [31], their role in there are unknown. Based on the present results, we propose that the functions of Abcg5/8 in the colon may be different from those in the small intestine and that these transporters have a protective role in colitis, especially in cases with IL-33-mediated intestinal inflammation. Moreover, since *Abcg5/8* siRNAs were shown to cause a decrease in tight junction gene expression, we also speculate that ABCG5/8 have a capability to maintain tight junction molecules, while the resulting dysfunction impairs barrier functions in the colon, resulting in prolonged inflammation. Nevertheless, further mechanistic investigations are needed to examine these phenomena and clarify how colonic homeostasis is maintained by ABCG5/8.

Although our results clearly showed downregulation of ABCG5/8 in inflamed areas of colons of UC patients as compared with noninflamed areas and samples from healthy individuals, they are limited by lack of functional analysis findings providing direct evidence of a colonic IL-33-ABCG5/8 axis in human IBD. In addition, IBD is a heterogeneous disorder and the immunological features differ among individual patients. Future studies that investigate the role of colonic *ABCG5/8* in IBD patients may be needed to specify the characteristics of those most closely associated with an IL-33-mediated immune disorder.

In summary, we identified a possible mechanism underlying IL-33-mediated mucosal inflammation in the colon. Downregulation of ABCG5/8 molecules may be a key process in IL-33-related colitis in UC patients. We consider that our findings will lead to better treatment options for heterogenetic IBD patients shown to be susceptible to IL-33-mediated immune disorders.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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References

1. Sartor RB, Wu GD. Roles for Intestinal bacteria, viruses, and fungi in pathogenesis of inflammatory bowel diseases and therapeutic approaches. *Gastroenterology*. 2017;152:327–339.e4.
2. Maloy KJ, Powrie F. Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature*. 2011;474:298–306.
3. Khor B, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. *Nature*. 2011;474:307–17.
4. Schmitz J, Owyang A, Oldham E, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity*. 2005;23:479–90.
5. Lu J, Kang J, Zhang C, et al. The role of IL-33/ST2L signals in the immune cells. *Immunol Lett*. 2015;164:11–7.
6. Pastorelli L, Garg RR, Hoang SB, et al. Epithelial-derived IL-33 and its receptor ST2 are dysregulated in ulcerative colitis and in experimental Th1/Th2 driven enteritis. *Proc Natl Acad Sci USA*. 2010;107:8017–22.
7. Kobori A, Yagi Y, Imaeda H, et al. Interleukin-33 expression is specifically enhanced in inflamed mucosa of ulcerative colitis. *J Gastroenterol*. 2010;45:999–1007.
8. Beltrán CJ, Núñez LE, Díaz-Jiménez D, et al. Characterization of the novel ST2/IL-33 system in patients with inflammatory bowel disease. *Inflamm Bowel Dis*. 2010;16:1097–107.
9. Latiano A, Palmieri O, Pastorelli L, et al. Associations between genetic polymorphisms in IL-33, IL1R1 and risk for inflammatory bowel disease. *PLoS ONE*. 2013;8:e62144.
10. Oboki K, Ohno T, Kajiwarra N, et al. IL-33 is a crucial amplifier of innate rather than acquired immunity. *Proc Natl Acad Sci USA*. 2010;107:18581–6.
11. Sedhom MAK, Pichery M, Murdoch JR, et al. Neutralisation of the interleukin-33/ST2 pathway ameliorates experimental colitis through enhancement of mucosal healing in mice. *Gut*. 2013;62:1714–23.
12. Pushparaj PN, Li D, Komai-Koma M, et al. Interleukin-33 exacerbates acute colitis via interleukin-4 in mice. *Immunology*. 2013;140:70–7.
13. Seidelin JB, Coskun M, Kvist PH, et al. IL-33 promotes GATA-3 polarization of gut-derived T cells in experimental and ulcerative colitis. *J Gastroenterol*. 2015;50:180–90.
14. De Salvo C, Wang X-M, Pastorelli L, et al. IL-33 drives eosinophil infiltration and pathogenic type 2 helper T-cell immune responses leading to chronic experimental ileitis. *Am J Pathol*. 2016;186:885–98.
15. Duan L, Chen J, Zhang H, et al. Interleukin-33 ameliorates experimental colitis through promoting Th2/Foxp3⁺ regulatory T-cell responses in mice. *Mol Med*. 2012;18:753–61.
16. Groß P, Doser K, Falk W, et al. IL-33 attenuates development and perpetuation of chronic intestinal inflammation. *Inflamm Bowel Dis*. 2012;18:1900–9.
17. Sattler S, Ling G-S, Xu D, et al. IL-10-producing regulatory B cells induced by IL-33 (Breg(IL-33)) effectively attenuate mucosal inflammatory responses in the gut. *J Autoimmun*. 2014;50:107–22.

18. Schiering C, Krausgruber T, Chomka A, et al. The alarmin IL-33 promotes regulatory T-cell function in the intestine. *Nature*. 2014;513:564–8.
19. Lee MH, Lu K, Hazard S, et al. Identification of a gene, ABCG5, important in the regulation of dietary cholesterol absorption. *Nat Genet*. 2001;27:79–83.
20. Edelblum KL, Turner JR. The tight junction in inflammatory disease: communication breakdown. *Curr Opin Pharmacol*. 2009;9:715–20.
21. Plösch T, Bloks VW, Terasawa Y, et al. Sitosterolemia in ABC-transporter G5-deficient mice is aggravated on activation of the liver-X receptor. *Gastroenterology*. 2004;126:290–300.
22. Yu L, Hammer RE, Li-Hawkins J, et al. Disruption of Abcg5 and Abcg8 in mice reveals their crucial role in biliary cholesterol secretion. *Proc Natl Acad Sci USA*. 2002;99:16237–42.
23. Ortega-Cava CF, Ishihara S, Rumi MAK, et al. Epithelial toll-like receptor 5 is constitutively localized in the mouse cecum and exhibits distinctive down-regulation during experimental colitis. *Clin Vaccine Immunol*. 2006;13:132–8.
24. Mishima Y, Ishihara S, Oka A, et al. Decreased frequency of intestinal regulatory CD5+ B cells in colonic inflammation. *PLoS ONE*. 2016;11:e0146191.
25. Kusunoki R, Ishihara S, Tada Y, et al. Role of milk fat globule-epidermal growth factor 8 in colonic inflammation and carcinogenesis. *J Gastroenterol*. 2015;50:862–75.
26. Zhu J, Yang F, Sang L, et al. IL-33 aggravates DSS-induced acute colitis in mouse colon lamina propria by enhancing Th2 cell responses. *Mediators Inflamm*. 2015;2015:913041.
27. Alex P, Zachos NC, Nguyen T, et al. Distinct cytokine patterns identified from multiplex profiles of murine DSS and TNBS-induced colitis. *Inflamm Bowel Dis*. 2009;15:341–52.
28. Shawki A, McCole DF. Mechanisms of Intestinal epithelial barrier dysfunction by adherent-invasive *Escherichia coli*. *Cell Mol Gastroenterol Hepatol*. 2017;3:41–50.
29. Saatian B, Rezaee F, Desando S, et al. Interleukin-4 and interleukin-13 cause barrier dysfunction in human airway epithelial cells. *Tissue Barriers*. 2013;1:e24333.
30. Chalubinski M, Wojdan K, Luczak E, et al. IL-33 and IL-4 impair barrier functions of human vascular endothelium via different mechanisms. *Vascul Pharmacol*. 2015;73:57–63.
31. Mutch DM, Anderle P, Fiaux M, et al. Regional variations in ABC transporter expression along the mouse intestinal tract. *Physiol Genomics*. 2004;17:11–20.