Original Article

The anti-inflammatory and protective role of interleukin-38 in inflammatory bowel disease

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(Received 4 August, 2021; Accepted 21 August, 2021; Released online in J-STAGE as advance publication 5 November, 2021)

Interleukin (IL)-38 exerts an anti-inflammatory function by binding to several cytokine receptors, including the IL-36 receptor. In this study, we evaluated IL-38 expression in the inflamed mucosa of patients with inflammatory bowel disease (IBD) and investigated its functions. IL-38 mRNA expression in endoscopic biopsy samples was evaluated using quantitative PCR. IL-38 protein expression was analyzed using immunohistochemical technique. Dextran sulfate sodium-induced colitis was induced in C57BL/6 background IL-38KO mice. The IL-38 mRNA and protein expression were enhanced in the active mucosa of ulcerative colitis, but not in Crohn's disease. The ratio of IL-36γ to IL-38 mRNA expression was significantly elevated in the active mucosa of UC patients. Immunofluorescence staining revealed that B cells are the major cellular source of IL-38 in the colonic mucosa. IL-38 dose-dependently suppressed the IL-36γ-induced mRNA expression of CXC chemokines (CXCL1, CXCL2, and CXCL8) in HT-29 and T84 cells. IL-38 inhibited the IL-36γ-induced activation of nuclear-factor kappa B (NF-κB) and mitogen-activated protein kinases in HT-29 cells. DSS-colitis was significantly exacerbated in IL-38KO mice compared to wild type mice. In conclusion, IL-38 may play an anti-inflammatory and protective role in the pathophysiology of IBD, in particular ulcerative colitis, through the suppression of IL-36-induced inflammatory responses.

***Key Words*: inflammatory bowel disease, ulcerative colitis, interleukin-38, interleukin-36**

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is a chronic and relapsing inflammatory disorder of the gastrointestinal tract. Although its precise etiology remains unknown, impaired cytokine expression has been reported to be involved in the pathogenesis of IBD.[1,2] Indeed, the biologic agents targeting the neutralization of pro-inflammatory cytokines, such as anti-TNF-α and anti-interleukin (IL)-12/23 p40 antibodies, have been clinically applied for IBD treatment. Thus, the investigation of the cytokine network in the intestinal mucosa is particularly important in understanding the pathophysiology of IBD and in developing a new therapeutic strategy.

The IL-1 family members play an important role in the cytokine network in inflammatory responses. Among them, IL-36 cytokines (IL-36α, IL-36β, and IL-36γ) have been reported to be associated with various inflammatory disorders, such as psoriasis, rheumatoid arthritis, and bronchial asthma.[3–6] Previous studies[4,7,8] have also reported that expression of IL-36α and IL-36γ is upregulated in the inflamed mucosa of IBD. IL-36 cytokines bind to a heterodimeric receptor consisting of the IL-36 receptor (IL-36R) subunit and the IL-1 receptor accessory protein (IL-1RAcP),[9,10] which are expressed in nonimmune cells in the colonic mucosa.[4,8,11] IL-36 cytokines induce C-X-C motif ligand (CXCL) chemokines through the activation of nuclear-factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs).[4,7]

IL-38, known as IL-1F10, belongs to the IL-1 family.[12] IL-38 shares 37% sequence homology with the IL-1R antagonist (IL-1Ra) and 43% homology with the IL-36R antagonist (IL-36Ra).[13] Due to homology to IL-36Ra, IL-38 can act as an IL-36 antagonist.[14] IL-38 mRNA is translated as a 152-amino acid (16.9 kD) precursor,[15] and N-terminal processing is considered essential for the full biological activity of IL-38.[16] It has been reported that IL-38 reduces IL-36-induced CXCL8 secretion from human peripheral mononuclear cells and keratinocytes.[14,17] In a mouse model of psoriasis, IL-38 inhibited IL-17 production from γδ T cells.[18] IL-38-gene knockout (IL-38KO) mice exhibited severe joint inflammation in a rheumatoid arthritis model.[19] These suggest the protective function of IL-38 in inflammatory settings. However, the precise role of IL-38 in the pathophysiology of IBD remains unclear.

Here, we report that the expression of IL-38 is upregulated in the active mucosa of UC patients. IL-38 suppressed the IL-36-induced CXC chemokine expression in intestinal epithelial cells. Dextran sulfate sodium (DSS)-induced experimental colitis was exacerbated in IL-38KO mice. These findings suggest an anti-inflammatory and protective role of IL-38 in the pathophysiology of UC.

Materials and Methods

Human Samples. Diagnosis of IBD was based on the clinical, endoscopic, and histopathological criteria. Demographic characteristics of patients are presented in Table 1. The IBD clinical activity was determined by the UC colitis activity[20] and Crohn's activity indexes.[21] The colonic biopsy specimens were obtained under colonoscopy from patients with UC and CD with written informed consents. All experiments were approved by the Ethics Committee at the Shiga University of Medical Science (permission number R2018-083).

Human colonic epithelial cell lines. The human colonic epithelial cell lines HT-29 and T84 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured according to the ATCC instructions. The recombinant proteins used in this study are listed in Supplemental Table 1*.

Quantitative real-time PCR. The relative expression levels of mRNA in the colonic epithelial cells were determined by quantitative RT-PCR. Briefly, the total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA). cDNA was synthesized using a first strand cDNA synthesis kit (Takara

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*See online. https://doi.org/10.3164/jcbn.21-104

doi: 10.3164/jcbn.21-104

Table 1. Demographic and basic characteristics of patients

	Normal	UC active	UC inactive	CD active	CD inactive
Number of samples	17	17	16	13	13
Age, years (range)	52 (38–72)	42 (20–74)	46.5 (21–62)	34 (24–40)	35 (20–69)
Female/Male	7/10	6/11	5/12	5/8	3/10
Disease duration, years (range)		6 (1–20)	4 (1–12)	5 (0–22)	5 (1–20)
Treatment, *n* (%)					
Mesalazine		14 (82.3)	15 (93.7)	12 (92.3)	13 (100)
Corticosteroids		6 (35.2)	3 (18.7)	1 (7.6)	1 (7.6)
Thiopurines		5 (29.4)	3 (18.7)	5 (38.4)	6 (46.1)
Anti-TNF		4 (23.5)	1 (6.2)	10 (76.9)	7 (53.8)
UC extent, *n* (%)					
Proctitis		3 (11.7)	4 (25)		
Left-sided colitis		7 (41.1)	8 (50)		
Pancolitis		7 (41.1)	4 (25)		
CD location, *n* (%)					
Ileal				6 (46.1)	8 (61.5)
Colonic				0 (0)	0 (0)
Ileocolonic				7 (53.8)	5 (38.4)
CD behavior, *n* (%)					
Non-stricturing and non-penetrating				5 (38.4)	6 (46.1)
Stricturing				5 (38.4)	6 (46.1)
Penetrating				3 (23.0)	1 (7.6)

Bio, Kusatsu, Japan). Real-time PCR was performed using a LightCycler 480 system (Roche Applied Science, Penzberg, Germany) or Thermal Cycler Dice Real Time System III (Takara Bio). The PCR primers used in this study are listed in Supplemental Table 2*. The values were normalized to GAPDH levels.

Nuclear and cytoplasmic protein extraction and immunoblot analysis. Nuclear proteins were extracted using a CelLytic NuCLEAR Extraction Kit (Sigma-Aldrich Co., St. Louis, MO) and cytoplasmic proteins were extracted according to the method described previously.[4] The samples were subjected to SDS-PAGE on a 10% gel under reducing condition and immunoblotting.[4] The antibodies used are listed in Supplemental Table 1*. Signal detection was performed using an enhanced chemiluminescence Western blot system (GE Healthcare, Little Chalfont, UK).

Immunohistochemical analysis. The formalin-fixed and paraffin-embedded sections were deparaffinized and incubated with Histo VT One (Nacalai Tesque, Kyoto, Japan) for antigen retrieval according to the manufacturer's instructions. After cooling, an anti-IL-38 antibody (Abcam, Cambridge, UK) was applied and incubated overnight at 4°C. DyLight 549-labeled anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA) was used as a secondary antibody. For double-staining procedures, anti-IL-38, anti-CD3 (Leica Biosystems, Buffalo Grove, IL), anti-CD19 (Santa Cruz Biotechnology, Dallas, TX), anti-CD68 (DAKO, Santa Clara, CA), and anti-myeloperoxidase (MPO) antibodies (R&D systems, Minneapolis, MN) were applied and incubated overnight at 4°C. Then, the slides were incubated with secondary antibodies conjugated to fluorescent dyes for 1 h at room temperature, visualizing the nuclei with 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI) (Vector Laboratories). Images were obtained with a confocal microscope TCS SP8 X (Leica Microsystems, Wetzlar, Germany). The antibodies used are listed in Supplemental Table 1*.

Dextran sulfate sodium-induced colitis in IL-38KO mice. IL-38KO mice with a C57BL/6N genetic background were kindly provided by Prof. Tomoaki Hoshino (Kurume University School of Medicine, Kurume, Japan).[19,22] All mice were kept under specific pathogen-free conditions. Eight to twelve-week-old females were treated with 2% DSS (molecular weight 36,000–50,000; MP Biomedicals, Santa Ana, CA) in drinking water for 4 days. Body weights of mice were monitored daily, and mice were euthanized if they lost more than 30% weight. After euthanizing mice by isoflurane overexposure, the colons were removed and fixed 10% neutral buffered formalin (Nacalai Tesque). Histological scores were evaluated by a previously validated scoring system.[23] All mice experiments were approved by the Research Center for Animal Life Science and Use Committee at the Shiga University of Medical Science (permission number 2018-4-5).

Statistical analysis. Statistical analyses were performed using GraphPad Prism software ver. 8 (GraphPad Software, San Diego, CA). The difference between the two groups was evaluated using a two-tailed Student's *t* test. For multiple group comparisons, statistical analysis was performed using a one-way ANOVA, followed by Sidak's post hoc tests. A $p<0.05$ difference was considered significant.

Results

The IL-38 mRNA expression was evaluated in the biopsy samples taken from healthy controls and IBD patients. A significant increase in IL-38 mRNA expression was detected in the active mucosa of UC patients as compare to the inactive mucosa and/or normal mucosa of healthy controls (Fig. 1A). However, this increase was not detected in the active mucosa of CD patients (Fig. 1A). Since IL-38 has been reported to act as an IL-36R antagonist,[14] we evaluated the ratio of IL-36 mRNA expression to IL-38 mRNA expression (IL-36/IL38 ratio) in the inflamed mucosa of UC patients. The IL-36α/IL-38 ratio tended to be increased in the active mucosa compared to the inactive mucosa but did not reach statistical significance (Fig. 1B). On the other hand, the IL-36γ/IL-38 ratio was significantly increased in the active mucosa compared to the inactive mucosa (Fig. 1C).

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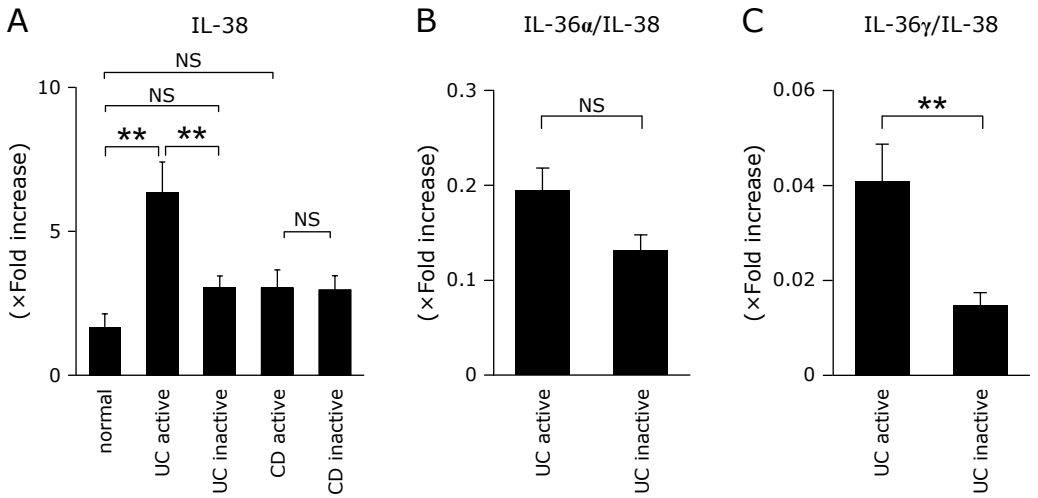

Fig. 1. IL-38 expression in the colonic mucosa of IBD patients. (A) Mucosal expression of IL-38 mRNA in healthy controls and IBD patients. IL-38 mRNA expression was normalized to GAPDH mRNA expression and is presented as fold-increase compared to the data of normal mucosa. (Healthy controls, $n = 17$; active UC, $n = 17$; inactive UC, $n = 16$; active CD, $n = 13$; inactive CD, $n = 13$). (B) The ratio of IL-36γ mRNA expression to IL-38 mRNA expression (IL-36α/IL-38 ratio). (C) The ratio of IL-36γ mRNA expression to IL-38 mRNA expression (IL-36γ/IL-38 ratio). Values are expressed as mean values ± SEM. $^{**}p<0.01$.

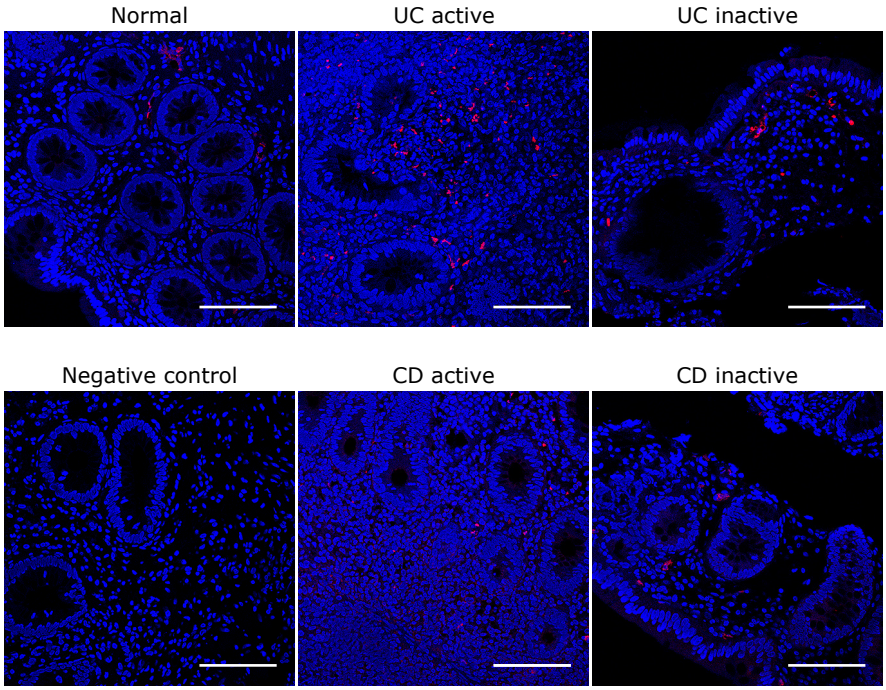

Fig. 2. Representative immunofluorescence images of IL-38 protein expression (red fluorescence) in colonic biopsy samples from healthy controls and IBD patients. Original magnification is ×200, and the scale bar indicates 100 μm. See color figure in the on-line version.

These observations indicate the dysregulated inflammatory responses mediated by IL-36 and IL-38 in the inflamed mucosa of UC patients.

The IL-38 protein expression was examined using immunohistochemical staining (Fig. 2). IL-38-expressing cells were markedly increased in the active mucosa of UC patients compared to the normal mucosa of healthy controls. This was not observed in the active mucosa of CD patients. These observations are compatible with the results of the mRNA levels. To characterize IL-38-expressing cells in the active mucosa of UC patients, we performed double immunofluorescence staining. Most of the IL-38-expressing cells were positive for CD19 (B cells) (Fig. 3A) but negative for CD3 (T cells) (Fig. 3B), CD68 (monocytes/macrophages) (Fig. 3C) and myeloperoxidase (MPO, neutrophils) (Fig. 3D). These indicate that B cells are the major cellular source of IL-38 in the colonic mucosa.

We have previously demonstrated that IL-36γ induces expression of CXC chemokines in intestinal epithelial cells.[4] So, we investigated the effects of IL-38 on the IL-36γ-induced CXC chemokine expression in intestinal epithelial cell lines (Fig. 4).

doi: 10.3164/jcbn.21-104

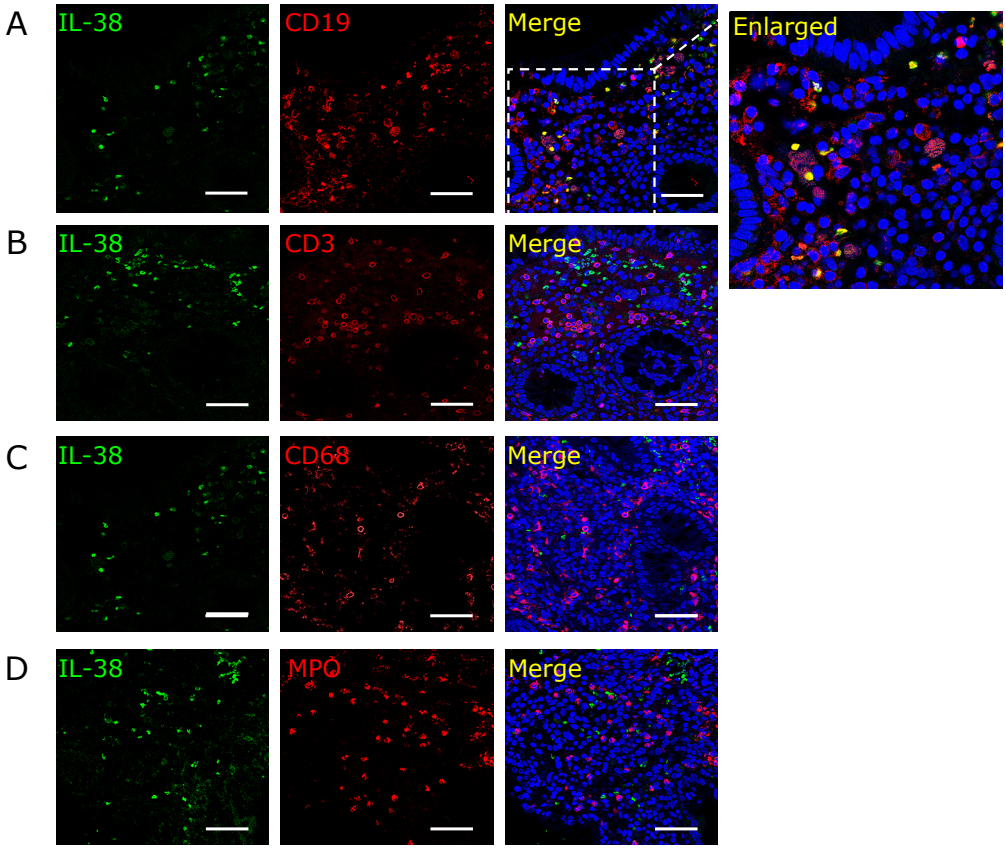

Fig. 3. Double immunofluorescence studies for IL-38 protein expression in the active mucosa of UC patients. IL-38 (green) and (A) CD19 (B cells, red), (B) CD3 (T cells, red), (C) CD68 (monocyte/macrophage, red), and (D) myeloperoxidase (MPO) (neutrophils, red). Double positive cells are detected as yellow in the merged panel. Original magnification is ×400, and the scale bar indicates 50 μm. See color figure in the on-line version.

In HT-29 and T84 cells, IL-38 dose-dependently suppressed the IL-36γ-induced CXCL1, CXCL2, and CXCL8 mRNA expression.

It has been reported that a signaling cascade downstream of the IL-36R complex results in the activation of MAPKs, NF-κB, and AP-1,[4,10,24] which regulate the expression of a number of inflammatory genes including CXCL chemokines. Using immunoblotting analysis, we investigated the effects of IL-38 on the IL-36γ-induced activation of MAPKs, NF-κB, and AP-1 in HT-29 cells. As shown in Fig. 5A, IL-38 dose-dependently suppressed IL-36-induced phosphorylation of MAPKs (p42/44 MAPK, p38 MAPK, and JNK) and IκBα. IL-38 also dose-dependently blocked the translocation of c-Jun (AP-1) and NF-κB p65 to the nucleus (Fig. 5B). These results indicate that IL-38 blocks the IL-36γ-induced intracellular signaling pathways.

We addressed the pathophysiological role of IL-38 in DSS-induced colitis model. IL-38KO mice were normal in body weight gain and showed no symptoms of colitis (Fig. 6A). Colitis was induced by oral administration of 2.0% DSS in drinking water for 4 days. Body weight loss on day 9 was significantly higher in IL-38KO mice than in wild type mice (Fig. 6A). Moreover, colon length was significantly shorter in IL-38KO mice than in wild type mice (Fig. 6B). There were no histological changes in the colonic mucosa of non-treated IL-38KO mice. However, the histological score was significantly higher in DSS-treated IL-38KO mice than in DSS-treated wild type mice (Fig. 6C and D). Thus, IL-38KO mice were highly susceptible to DSS-colitis, indicating a protective role of IL-38 in the colon.

Discussion

In this study, we demonstrate several new findings regarding IL-38, which may be involved in the pathophysiology of IBD, in particular UC. These include: (a) increased expression of IL-38 in the inflamed mucosa of UC patients, (b) B cells are the major cellular source of IL-38 in the colonic mucosa, (c) suppressive effects of IL-38 on the IL-36γ-induced CXC chemokine expression in colonic epithelial cells, (d) inhibitory effects of IL-38 on the IL-36γ-induced activation of MAPKs, NF-κB, and AP-1, (d) exacerbation of DSS-colitis in IL-38KO mice.

IL-1 family members consist of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-33, IL-18, IL-36α, IL-36β, and IL-36γ, and anti-inflammatory cytokines such as IL-1Ra, IL-36Ra, IL-37, and IL-38.[25] IL-38 exerts anti-inflammatory effects by antagonizing IL-36R and IL-1R[15] and has been reported to be associated with various inflammatory disorders, including psoriasis, rheumatoid arthritis, bronchial asthma, primary Sjögren's syndrome, and systemic lupus erythematosus.[17–19,22,26–28] In this study, we demonstrated a significant increase in IL-38 expression in the active mucosa of UC patients, but this was not observed in CD patients. We have previously reported that IL-36γ expression is specifically enhanced in the active mucosa of UC patients, but not in CD patients.[4] Furthermore, we found a significant elevation of the IL36γ/IL38 ratio in the active mucosa of UC patients. These findings suggest that the imbalance of pro-inflammatory IL-36 and anti-inflammatory IL-38 may contribute to the pathological condition characteristic to UC.

IL-36 cytokines are regarded as pro-inflammatory cytokines, since their binding to IL-36R stimulates intracellular signaling

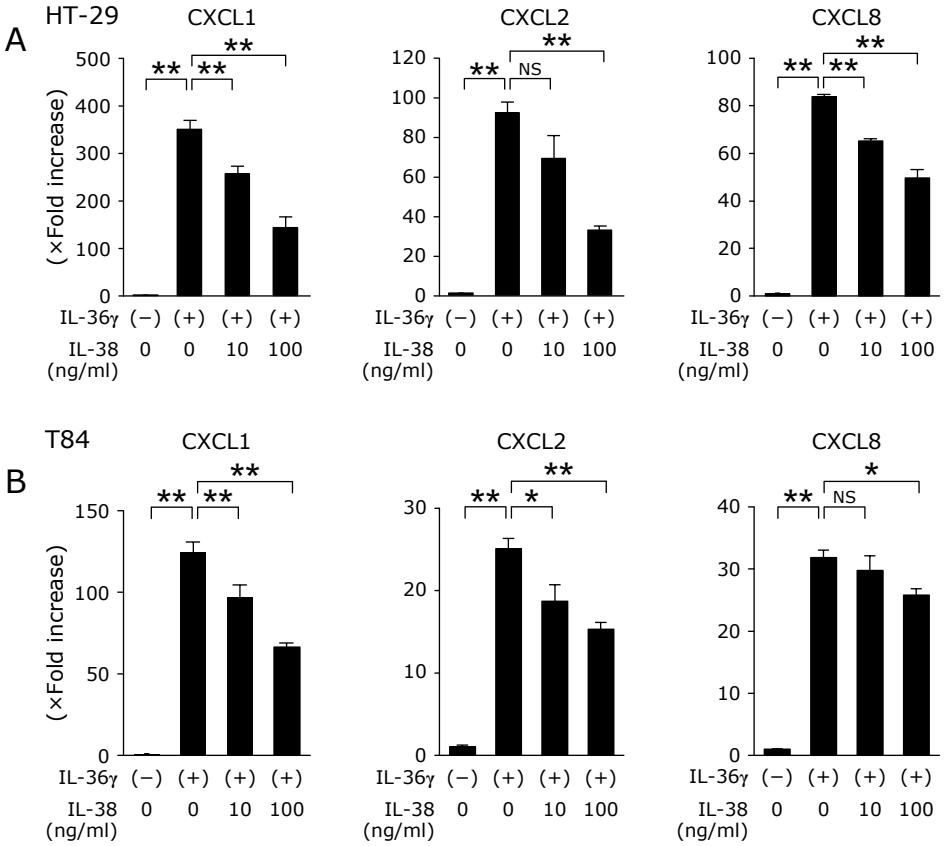

Fig. 4. Inhibitory effects of IL-38 on IL-36γ-induced CXC chemokine mRNA expression in intestinal epithelial cells. (A) HT29 cells were stimulated with IL-36γ (100 ng/ml) for 12 h with or without IL-38. The mRNA levels of CXC chemokines (CXCL1, CXCL2, and CXCL8) were evaluated by quantitative PCR. (B) T84 cells were stimulated with IL-36γ (100 ng/ml) for 12 h with or without IL-38. The mRNA levels of CXC chemokines (CXCL1, CXCL2, and CXCL8) were evaluated by quantitative PCR. Values are expressed as mean values ± SEM ($n = 4$). *$p<0.05$, **$p<0.01$.

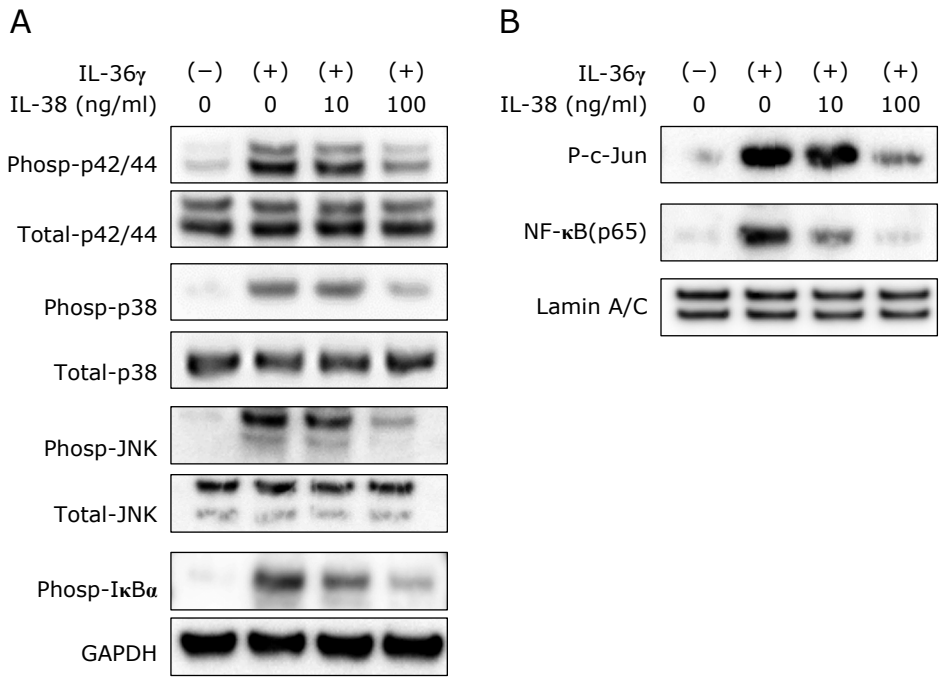

Fig. 5. Inhibitory effects of IL-38 on IL-36γ-induced activation of MAPKs, NF-κB, and c-Jun (AP-1). The HT-29 cells were stimulated with IL-36γ (100 ng/ml) for 15 min with or without IL-38. (A) The phosphorylation of MAPKs and IκBα was evaluated by immunoblot analyses. The data are representative of two independent experiments. (B) The translocation of NF-κB (p65) and AP-1 (c-Jun) into the nucleus was evaluated by immunoblot analysis using nuclear proteins. Lamin A/C was used as a loading control. The data are representative of two individual experiments.

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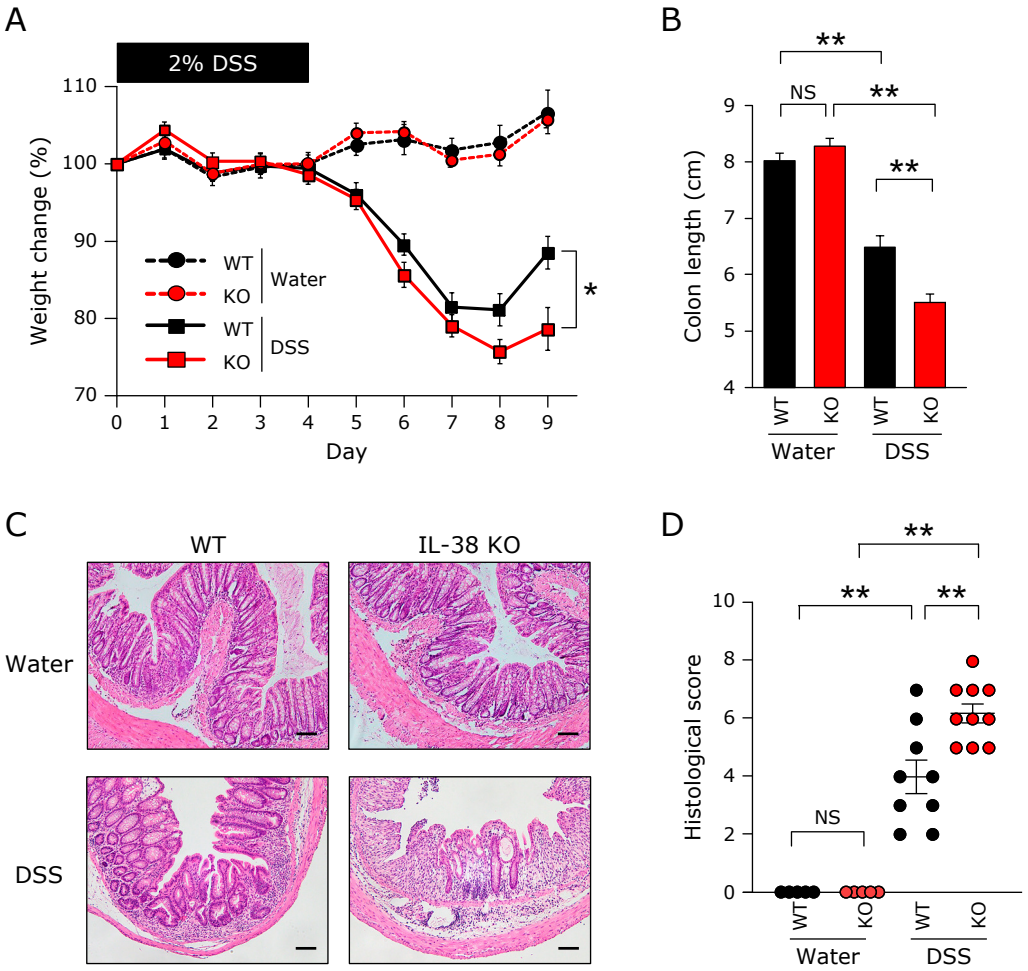

Fig. 6. DSS-colitis in C57BL/6 background IL-38KO mice. Experimental colitis was induced by oral administration of 2% w/v DSS in drinking water for 4 days and followed by 5 days water in wild type (WT) mice and IL-38KO mice. (A) Changes in relative body weight ($n = 8$ in each group). (B) Colon length. (C) Representative microscopic pictures of the distal colon (original magnification is ×200). (D) Histological score. Values are expressed as mean values ± SEM. *p<0.05, **p<0.01. WT, wild type; KO, IL-38KO.

pathways which leads to the activation of NF-κB and MAPKs.[4,10,29] In this study, IL-38 blocked the IL-36γ-induced activation of MAPKs, NF-κB and AP-1, and suppressed the following induction of CXC chemokines (CXCL1, CXCL2, and CXCL8) in the intestinal epithelial cells. This may be induced by a homology-based antagonistic effect of IL-38 on the IL-36 binding to IL-36R. CXCL1, CXCL2, and CXCL8 are strong chemoattractant and activator of neutrophils. Neutrophils are responsible for the first defense against invading pathogens via phagocytosis and killing by producing reactive oxygen species,[30] but excessive infiltration and accumulation of neutrophils leads to profound tissue damage and prolonged inflammation.[30] So, the suppressive effect of IL-38 on the IL-36γ-induced CXCL chemokine expression is considered as one of anti-inflammatory and protective properties of IL-38. We also demonstrated the protective role of IL-38 using DSS-colitis model in IL-38KO mice. DSS-colitis was significantly exacerbated in IL-38KO mice. IL-38KO mice were highly susceptible to DSS-colitis. Our observations are consistent with the findings of a recent report by Xie *et al.*[31] showing that recombinant IL-38 injection attenuated DSS-colitis.

Like IL-36 and IL-38, we have previously demonstrated that expression of some cytokines such as IL-33, IL-13, IL-24, and eotaxin-3 are more strongly expressed in UC mucosa than in CD mucosa.[32–34] These may characterize a different pathophysiology 3 from the bottom between UC and CD. In this study, we showed the mRNA expression of IL-38 was significantly elevated in the active mucosa of UC but not CD patients, suggesting that IL-38 could play different roles in UC and CD. The precise mechanisms underlying the different cytokine signatures between UC and CD should be investigated in the future.

Previous studies[12,35,36] have reported that IL-38 is secreted by various cell types, such as peripheral blood mononuclear cells, fibroblasts, B cells, keratinocytes, and various immune cells. However, the cellular source of IL-38 in the intestinal mucosa remains unclear. In this study, we found that IL-38 was mainly expressed in B cells in the inflamed mucosa of UC patients. IL-38 expression was not detected in other cell types such as T cells, monocytes/macrophages and neutrophils. B cells have been reported to secret some kinds of cytokines.[37] For example, Mizoguchi *et al.*[38] previously reported that a B cell subgroup in the intestinal mucosa can secrete IL-10 and suppresses progression of intestinal inflammation. The IL-10-secreting B cells are now termed regulatory B cells, and the homology between regulatory B cells and IL-38-secreting B cells should be clarified in the future.

In conclusion, IL-38 expression is enhanced in the active mucosa of UC patients, but not in CD patients. In the colonic mucosa, CD19-positive B cells were the major cellular source of IL-38. IL-38 suppressed IL-36γ-induced inflammatory responses

in intestinal epithelial cells, and DSS-colitis was exacerbated in IL-38KO mice. Thus, IL-38 functions as an anti-inflammatory cytokine and acts protectively in DSS-colitis. However, further studies are required to define the molecular mechanism by which IL-38 and IL-36 are specifically induced in active UC patients. Characterization of IL-38-secreting B cell subsets should also be investigated. The findings in this study suggest that the IL-36-IL-38 axis may be a new therapeutic target for treating IBD.

Acknowledgments

This work was supported by the Japan Agency for Medical Research and Development (AMED) under grant number JP20gm1010008h9904 (AA), and in part by a Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (JSPS KAKENHI) under grant number 19K08811(MK), 19K23844 (MO), 19K08637 (TH), and 18K08002(AA), and in part by Health and Labor Sciences Research Grants for Research on Intractable Diseases from the Ministry of Health, Labor and Welfare of Japan under grant number 20FC1037 (AA).

Conflict of Interest

AA received lecture fee from Janssen, Takeda, AbbVie, Tanabe-Mitsubishi. All other authors declare that they have no conflict of interest in this study.

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