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Interleukin-38 is elevated in inflammatory bowel diseases and suppresses intestinal inflammation

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ABSTRACT

There has been no report investigating the role of IL-38 in inflammatory bowel diseases (IBD). Therefore, we investigated the expression of IL-38 in IBD patients and its role in regulating intestinal inflammation. The levels of IL-38 were significantly elevated in the intestine of IBD patients and DSS-induced colitis mice. Immunofluorescence analysis revealed that B cell, not macrophage or T cell, was the source of IL-38 in the intestine. We found that rIL-38 treatment significantly attenuated DSS-induced colitis, including alleviation of weight loss, disease activity index, macroscopic changes and histological damage of colon, along with lower levels of IL-1β and TNF-α. In vitro, rIL-38 significantly decreased the expression of proinflammatory cytokines in LPS-stimulated RAW 264.7 cells and BMDM. This is the first study suggesting that IL-38 may have a protective effect in IBD, which inhibits the production of proinflammatory cytokines from macrophages. IL-38 may represent a promising therapeutic strategy in IBD.

1. Introduction

Inflammatory bowel diseases (IBD), mainly composed of ulcerative colitis (UC) and Crohn's disease (CD), are characterized by a broadly dysregulated immune response, including innate and adaptive immunity [1]. Cytokines mediating the crosstalk between innate and adaptive immunity are key effectors of both normal homeostasis and chronic inflammation in the gut [2]. Overproduction of proinflammatory cytokines has been reported to be concerned with the onset and persistence of the intestinal inflammation in IBD, such as IL-1β and TNF-α, etc [3]. Several monoclonal antibodies targeting some crucial cytokines have been approved and widely used for IBD therapy, including anti-TNFs (e.g., Infliximab, Etanercept, Adalimumab, etc.) and anti-IL-12/23 p40 (i.e., Ustekinumab) [4]. Collectively, cytokines are not only key players in the pathogenesis of IBD but also are viable therapeutic targets.

IL-38, also known as IL-1F10 or IL-1HY2, has recently been discovered as a new member of the IL-1 family (IL-1F). IL-1F is a group of cytokines similar to IL-1 and contains 11 members, which are termed as IL-1α, IL-1β, IL-1Ra, IL-18, IL-33, IL-36α, IL-36β, IL-36γ, IL-36Ra, IL-37 and IL-38, all playing significant roles in inflammation and immune responses. The IL-38 gene is situated on chromosome 2p13, adjacent to IL-1Ra and IL-36Ra [5]. Sequence analysis indicated that the IL-38 gene has 41% homology with IL-1Ra, 43% homology with IL-36Ra, and lower homology with other IL-1F members [5,6]. The primary translated product is a precursor of IL-38, which are 152 amino acids in length and 16.9kD molecular in mass [7]. Previously, IL-38 has been reported to be expressed in the basal epithelia of skin and in proliferating B cells of tonsils [8]. It is also found in spleen, thymus, placenta, heart, lung, fetal liver and so on [5].

It is well known that a large proportion of IL-1F cytokines are proinflammatory and essential to inflammation and auto-immunity [9]. As a cytokine sharing sequence homology with inhibitory cytokines such as IL-1Ra and IL-36Ra, IL-38 has currently been verified to exert anti-inflammatory effects. Dinarello et al found that IL-38 can downregulate the expression of Th17 related cytokines of peripheral blood

Abbreviations: IBD, inflammatory bowel disease; CD, crohn's disease; UC, ulcerative colitis; Ctrl, control; BMDM, bone marrow derived macrophage; IL, interleukin; IL-1F, IL-1 family; TNF-α, tumor necrosis factor-α; C-C motif, chemokine; CCL, ligand; DSS, dextran sulphate sodium; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; DAI, disease activity index; RT-qPCR, quantitative reverse transcription polymerase chain reaction; WB, western blotting; ELISA, enzyme-linked immunosorbent assay

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mononuclear cells (PBMC) from healthy donors stimulated by *C. albicans* [10]. Several studies demonstrated that IL-38 can reduce the production of IL-6 and IL-8 in LPS stimulated-macrophages and PBMC [11–13]. In experimental mouse models of arthritis, IL-38 limited macrophage infiltration into synovium and inhibited the secretion of Th17 related cytokines [10,12,13]. In additions, IL-38 has been identified as one of 18 markers connected with elevated C-reactive protein (CRP) levels in serum, based on a genome-wide association study (GWAS) involving 66,185 individuals [14], implying IL-38 may play an important role in inflammatory course.

As a poorly characterized IL-1F cytokine, IL-38 has raised general interest of its biological functions worldwide and several studies have shown that IL-38 is involved in the pathogenesis of many autoimmune diseases, including psoriasis [15,16], systemic lupus erythematous (SLE) [11], spondylitis ankylopoietica (AS) [17], rheumatoid arthritis (RA) [15], hidradenitis suppurativa (HS) [18], primary Sjögren syndrome [19], and gestational diabetes [20]. However, the role of IL-38 in IBD remains unclear. In this study, we explored the expression of IL-38 in IBD patients and mouse model, and then identified the cellular source of IL-38, as well as its role and regulatory mechanisms in a mouse model of DSS-induced acute colitis.

2. Materials and methods

2.1. Patients and samples

In this study, colon biopsy specimens were obtained from patients with CD (n = 30), UC (n = 31) and controls (n = 38). Serum specimens were harvested from patients with CD (n = 46), UC (n = 40) and controls (n = 43). These samples were collected from September 2017 to August 2018 at the department of gastroenterology of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. Those IBD patients were diagnosed comprehensively according to their clinical manifestations, radiologic examinations, endoscopic images, and pathological findings, as well as the exclusion of infectious colitis and systemic diseases. Clinical disease activity was assessed by Crohn's disease activity index (CDAI) [21] or Mayo score activity index [22]. The control group was recruited from patients with intestinal polyps, hemorrhoids or physical examinations, with normal colonoscopy. Clinical characteristics of the participants are summarized in Table 1.

This study was approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology. It was carried out in accordance with the principles of the Helsinki Declaration and the written informed consent of all participants was obtained before enrollment.

2.2. Mice

Male C57BL/6 mice, 8 weeks (22–24 g), were purchased from Beijing HFK Biotechnology Co., LTD (Beijing, China), and then raised at the animal facility of Tongji Medical College. Under specific pathogen-free conditions, the mice were free to obtain sterile water and autoclaved food. The mice were kept adaptable for one week before being used in the experiment. Animal researches were approved by the Animal Management and Use Committee of Huazhong University of Science and Technology.

2.3. Induction of DSS-induced colitis and intervention of IL-38 recombinant protein

C57BL/6 mice were administered with 2.5% dextran sulfate sodium (DSS) (MP Biomedicals, USA) in their drinking water for 7 days to induce acute colitis. Simultaneously, the animals were intraperitoneally injected with either mouse IL-38 recombinant protein (1 μg in 400 μL PBS/mouse) or equivolumetric PBS as control at 0, 1, 3, 5 and 7 day. Murine recombinant IL-38 protein (aa 3–152) was obtained from AdipoGen (Listal, Switzerland). The DSS-induced process and dosage regimen of IL-38 recombinant protein were presented in Fig. 4A. Body weight loss, stool consistency and bloody stools were recorded daily, and disease activity index (DAI) was calculated according to the calculation method defined by Cooper [23]: body weight loss (0, none; 1, 1–5%; 2, 6–10%; 3, 11–20%; 4, > 20%), stool consistency (0, normal; 2, loose stool; 4, diarrhea), and bloody stools (0, negative; 2, fecal occult blood test positive; 4, gross bleeding). After mice were executed on the 8th day, colons, spleen and mesenteric lymph nodes were obtained and stored for further analysis.

2.4. Quantitative real-time PCR

According to the manufacturer's instructions, total RNA was extracted using the RNAiso plus (Takara Bio Inc., Kusatsu, Shiga, Japan). Its quantity and quality were measured by a NanoVue spectrophotometer (GE Healthcare, Piscataway, NJ), with 260/280 ratio of 1.8–2.0. Equal amount of total RNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent Kit (Perfect Real-Time, Takara). Gene expression was determined by quantitative real-time PCR using SYBR® Premix Ex Taq™ (Tli RNaseH Plus, Takara) with the LightCycler®

Table 1
Clinical characteristics of the participants.

	Biopsy samples			Blood samples		
	Ctrl	CD	UC	Ctrl	CD	UC
Number	38	30	31	43	46	40
Age	35 (24–62)	23 (15–62)	44 (23–69)	33 (22–64)	24 (16–59)	45 (22–70)
Sex (Male/Female)	22/16	18/12	17/14	20/23	26/20	23/17
Disease activity (A/R)		25/5	29/2		21/25	28/12
Disease extent						
Small intestine		7			9	
Colonic		3			5	
Ileocolonic		20			32	
Proctitis			4			8
Left sided			12			18
Pancolitis			15			24
Current therapy						
5-Aminosalicylates		5	26		13	34
Steroids		2	11		6	16
Azathioprine		7	1		5	2
Biologics		19	0		29	0
No treatment		2	1		1	0

Table 2
The primers sequences used for RT-qPCR.

Gene	Forward	Reverse
Human IL-38	5′-TTATCCTTGTGGGCTCAGTT-3′	5′-AATCCGTTCCCTTGGCTTTT-3′
Human GAPDH	5′-GGAGCGAGATCCCTCCAAAAT −3′	5′-GGCTGTTGTCATACTTCTCATGG −3′
Mouse IL-38	5′-GGGAGATCCTGTTGCAGACAA-3′	5′-GGCCAAGCCTCTGTTAGGAAGTA-3′
Mouse IL-1β	5′-CCTCGTGCTGTCGGACCCATA-3′	5′-CAGGCTTGTGCTCTGCTTGTGA-3′
Mouse IL-6	5′-TAGTCCTTCCTACCCCAATTTCC-3′	5′-TTGGTCCTTAGCCACTCCTTC-3′
Mouse TNF-α	5′-CCTGTAGCCCACGTCGTAG-3′	5′-GGGAGTAGACAAGGTACAACCC-3′
Mouse IL-10	5′-CTTACTGACTGGCATGAGGATCA-3′	5′-GCAGCTCTAGGAGCATGTGG-3′
Mouse CCL-2	5′-CCCCAGTCACCTGCTGTTAT-3′	5′-GAGTTTGGGTTTGCTTGTCC-3′
Mouse CCL-7	5′-CCTGGGAAGCTGTTATCTTCAAG-3′	5′-CCTCCTCGACCCACTTCTGA-3′
Mouse IL-17	5′-CAGCAGCGATCATCCCTCAAAG-3′	5′-CAGGACCAGGATCTCTTGCTG-3′
Mouse IL-22	5′-ATGAGTTTTTCCCTTATGGGGAC-3′	5′-GCTGGAAGTTGGACACCTCAA-3′
Mouse IL-23	5′-CAGCAGCTCTCTCGGAATCTC-3′	5′-TGGATACGGGGCACATTATTTTT-3′
Mouse Foxp3+	5′-GTGGGCACGAAGGCAAAG-3′	5′-CCTTGTTTTGCGCTGAGAGTCT-3′
Mouse RORγt	5′-TGCGACTGGAGGACCTTCTAC-3′	5′-TCACCTCCTCCCGTGAAAAG-3′
Mouse TGF-β	5′-TGGAGCAACATGTGGAACTC-3′	5′-CAGCAGCCGGTTACCAAG-3′
Mouse β-actin	5′-AGTGTGACGTTGACATCCGTA-3′	5′-GCCAGAGCAGTAATCTCCTTCT-3′

480 System (Basel, Switzerland). The amplification conditions were as follows: pre-denaturation at 95 °C 10 min; and 40 cycles of denaturing at 95 °C 30sec, annealing at 60 °C 1 min, and extending at 72 °C 30sec for each cycle. Gene expression was standardized by human GAPDH gene or mouse β-actin gene. The relative levels of the target gene were determined by using the 2-ΔΔCt method. The primers used for analysis are shown in Table 2.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The levels of serum IL-38 were measured by using for human IL-38 quantikine ELISA kit (R&D Systems, Minnesota, USA) or murine IL-38 quantikine ELISA kit (R&D Systems, Minnesota, USA), being performed according to the manufacturer's instructions. The standard curve with four-parameter logistic (4-PL) curve-fit be applied to determine the serum sample concentration. The quantitative range of human IL-38 quantikine ELISA kit was between 31.3 and 2000 pg/ml. The quantitative range of murine IL-38 quantikine ELISA kit was between 250 and 16000 pg/ml.

2.6. Immunohistochemical staining (IHC)

Colon tissues were obtained, fixed with 4% paraformaldehyde, and embedded into paraffin blocks. Paraffin slices were dewaxed in xylene and rehydrated through a graded series of alcohol. Antigen retrieval was performed by microwave irradiation in 10 mM citrate buffer for 20 min. The sections were incubated with 3% hydrogen peroxide for 15 min to quench the endogenous peroxidase. After being washed three times with PBS, the sections were blocked with 10% nonimmunized goat serum for 30 min, and then incubated with Rabbit polyclonal to IL-38 antibody (1:300 dilution; Abcam, Cambridge, UK) overnight at 4°C. After being washed with PBS, the sections were incubated with horseradish peroxidase-conjugated secondary antibody (1:200 dilution; AntGene, Wuhan, China) for 30 min at room temperature. Subsequently, the sections were detected by using DAB staining (AntGene, Wuhan, China).

2.7. Western blotting (WB)

Extracted protein samples from murine colon tissues were electrophoretically separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then blotted onto polyvinylidene difluoride membranes. After placed into blocking buffer (8% non-fat milk in Tris-buffered saline with 0.1% Tween-20) for 1 h at room temperature, the membranes were incubated with an anti-IL-38 antibody (1:1000 dilution; Abcam, Cambridge, UK) at 4°C overnight. An anti-GAPDH antibody (1:2000 dilution; AntGene, Wuhan, China) was considered as internal controls. After being washed with TBST, the membranes were incubated with corresponding appropriate horseradish peroxidase-conjugated secondary antibodies (1:2000 dilution; AntGene, Wuhan, China) for 1 h at room temperature, subsequently being detected with enhanced chemiluminescence (ECL) (Absin, Shanghai, China) and exposure equipment (Bio-Rad, California, USA). Densitometric band intensity was acquired using the AlphaView Software (ProteinSimple, California, USA).

2.8. Immunofluorescence

To localize the expression of IL-38, 4% formalin-fixed and paraffin-embedded colonic sections were stained by immunofluorescence. The paraffin slices were dewaxed in xylene and rehydrated through a graded series of alcohol. Antigen retrieval was performed by using an electric voltage cooker in citrate buffer (pH = 6) for 3 min. Sections were permeated in PBS with 0.3% Triton-100 for 30 min at room temperature, being thoroughly cleaned with PBS. The sections were blocked with 10% goat serum for 30 min, and then incubated overnight with anti-IL-38 antibody (1:100 dilution; Abcam, ab180898, Cambridge, UK) and anti-CD3 antibody (1:150 dilution; Abcam, ab110898, Cambridge, UK), anti-CD19 antibody (1:50 dilution; Abcam, ab25232, Cambridge, UK) or with anti-CD68 antibody (1:100 dilution; Abcam, ab201340, Cambridge, UK) at 4°C. The next day, the sections were incubated IL-38 with corresponding fluorescent secondary antibody (1:200 dilution; Alexa Fluor 594, AntGene, Wuhan, China) and CD68, CD3 or CD19 with corresponding fluorescent secondary antibody (1:200 dilution; Alexa Fluor 488, AntGene, Wuhan, China) for 1 h at room temperature. After being counterstained with DAPI (AntGene, Wuhan, China), the location of IL-38 expression was detected by a Laser scanning confocal microscope (Nikon, Tokyo, Japan).

2.9. Cell cultures

Murine bone marrow-derived macrophages (BMDM) were obtained according to the procedure as described previously [24]. Briefly, bone marrow mononuclear cells were harvested by flushing the femurs and tibias of male C57BL/6 mice with PBS containing 3% fetal bovine serum (FBS, Gibco, Massachusetts, USA), following by culturing isolated mononuclear cells with RPMI 1640 medium (Gibco, Massachusetts, USA) supplemented with 10% FBS and 10 ng/ml M-CSF (PEPROTECH, New Jersey, USA) for 7 days. Non-adherent cells were removed on the 7th day, and adherent cells were collected for the following experiments.

Murine macrophages cell line (RAW 264.7) were cultured in Dulbecco's Modified Eagle's Medium (Gibco, Massachusetts, USA) supplemented with 10% FBS and 100U/mL penicillin-streptomycin (Gibco,

Massachusetts, USA). After reaching 80–90% confluence, the cells were dislodged from the flask substrate with a cell scraper. Cells were incubated in cell incubator (37 °C and 5% CO2).

For the analysis of cytokines production, RAW 264.7 cells and BMDM were split into 12-well culture plates and cultured for 24 h. Subsequently, cells were intervened with 100 ng/ml LPS and 100 ng/ml IL-38 recombinant protein for 12 h and were collected for further analysis.

2.10. Statistical analysis

SPSS 13.0 and GraphPad Prism 5.0 were used for statistical analysis. Data were presented as mean ± SEM. Statistical comparisons were analyzed using the unpaired 2-tailed Student's *t* test or Mann–Whitney *U* test as indicated. The association between serum IL-38 levels and CRP was analyzed with Spearman's correlation coefficients (r). It was considered as statistically significant differences when $p < 0.05$.

3. Results

3.1. Expression of IL-38 in patients with IBD

Recent studies have shown that IL-38 is associated with the pathogenesis of various autoimmune diseases, such as systemic lupus erythematosus [11] and rheumatic diseases [13]. Therefore, we investigated whether IL-38 participated in the development of IBD. Expression of IL-38 mRNA was identified by RT-qPCR in colon samples obtained from UC patients, CD patients and controls, indicating that IL-38 mRNA expression was significantly higher in UC and CD patients than that in controls (Fig. 1A). In addition, we further assessed the expression of IL-38 in the human intestinal mucosa by immunohistochemistry. As the representative images presented in Fig. 1D, markedly increased IL-38$^+$ cells were found infiltrated in the lamina propria from patients with UC and CD as compared with controls.

To further explore the level of systemic IL-38, we measured IL-38 expression in serum of IBD patients and controls. We identified no statistical difference in serum IL-38 levels between CD or UC patients and controls ($p = 0.197$ and $p = 0.805$, respectively) (Fig. 1B). The mean serum IL-38 concentrations were 7.09 pg/ml (range 0–93 pg/ml) in CD patients and 3.27 pg/ml (range 0–79 pg/ml) in UC patients, which were detectable in 7 of 46 CD samples (15.2%) and 3 of 40 UC samples (7.5%). In contrast, IL-38 concentrations were mean 1.19 pg/ml (range 0–31 pg/ml) and only detectable in 2 of 43 controls (5%). Additionally, there was no statistically significant correlation between serum IL-38 and CRP levels (Fig. 1C, $r = -0.210$; $p = 0.274$).

3.2. Expression of IL-38 in DSS-induced acute experimental colitis mice

Boutet et al reported that expression of IL38 mRNA increased in the colon of DSS induced colitis [15]. We confirmed the results and further identified the localization of IL-38 positive cells in colon, shown in Fig. 2. IL-38 mRNA level in colon, spleen and mesenteric lymph node was analyzed by RT-qPCR, and the results suggested that IL-38 mRNA level was distinctly higher in inflamed colon than that in noninflamed colon, while no significant differences were indicated in spleen or mesenteric lymph node between DSS-treated and normal control mice (Fig. 2A). The increasing of IL-38 expression in the colon of DSS mice was also confirmed by western blot analyses (Fig. 2B). Furthermore, immunohistochemistry showed that IL-38$^+$ cells in the colon of DSS mice remarkably increased compared with control mice with a

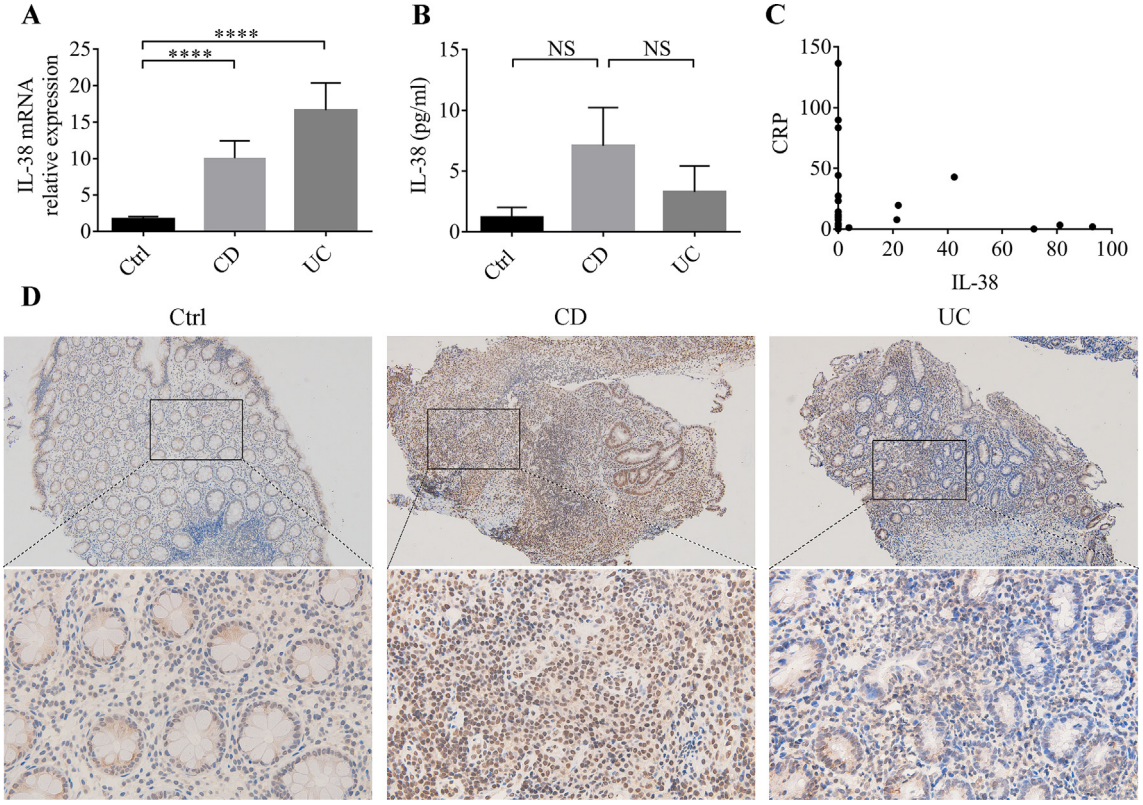

Fig. 1. Expression of IL-38 in patients with IBD. (A) Relative expression of IL-38 mRNA in colonic biopsies from controls or IBD patients was determined by RT-qPCR. Ctrl (n = 38), CD (n = 30), and UC (n = 31). Gene expression was standardized by GAPDH gene in each group. (B) Level of IL-38 was determined in serum from controls or IBD patients by ELISA. Ctrl (n = 43), CD (n = 46), and UC (n = 40). (C) Correlation between serum IL-38 and CRP (n = 29). And (D) IL-38 expression detected by immunohistochemistry in colonic tissue from controls or IBD patients. Original magnification × 100 (top) and × 400 (bottom). Statistical analysis was performed by the Mann–Whitney *U* test. NS, $P > 0.05$, ***$P < 0.001$ and ****$P < 0.0001$. Ctrl, control; CD, Crohn's disease; UC, ulcerative colitis; RT-qPCR, quantitative real-time PCR; ELISA, enzyme linked immunosorbent assay.

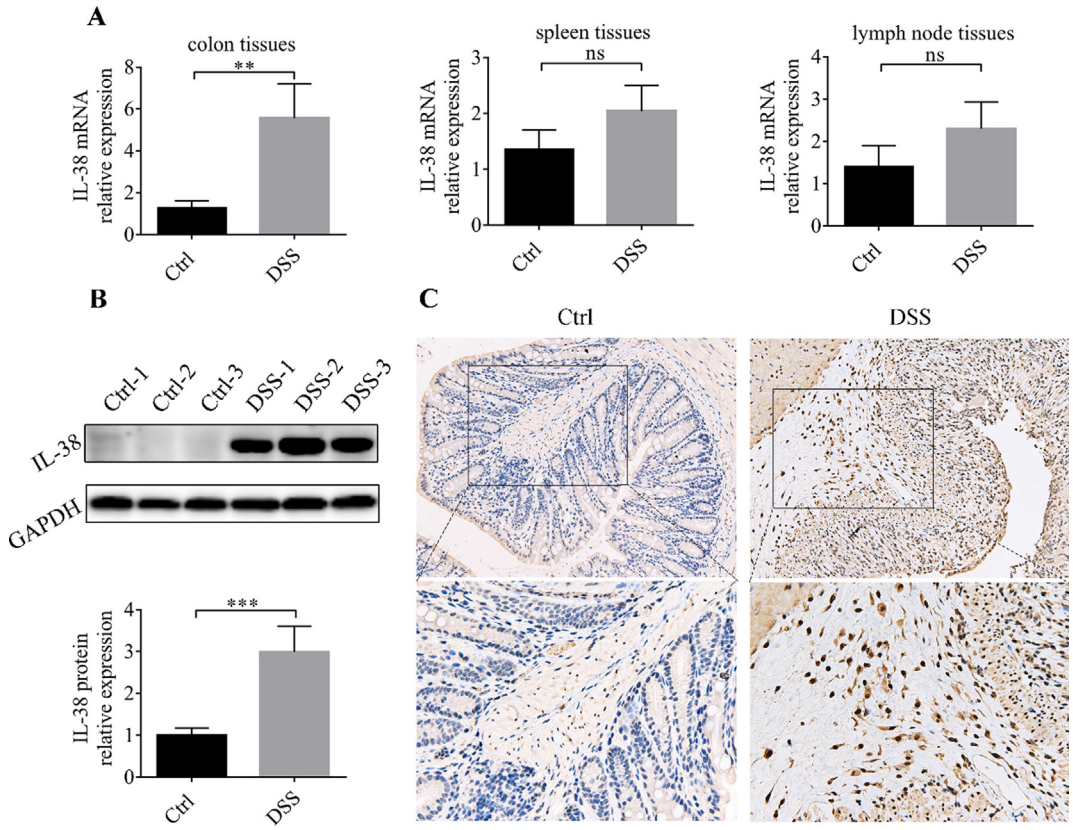

Fig. 2. Expression of IL-38 in DSS-induced acute experimental colitis mice. To induce colitis, mice were challenged with 2.5% DSS in drinking water for 7 days. (A) Relative expression of IL-38 mRNA in colon, spleen and mesenteric lymph node tissue of DSS-induced colitis mouse model and control group was determined by RT-qPCR. Results are normalized to β-actin gene. (B) Expression levels of IL-38 protein in colonic tissue was determined by Western blot (top) and relative IL-38 expression as quantified by densitometry after normalization to GAPDH (bottom). And (C) Representative immunohistochemistry images of IL-38 protein in colonic tissue from C57BL/6 mouse model compared with the control group. Original magnification ×100 and ×400. Data are represented as mean ± SEM; n = 6–8 for each group. Statistical analysis were performed by the Mann–Whitney *U* test. NS, $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$. RT-qPCR, quantitative real-time PCR.

distribution pattern throughout the colon wall, especially in mucosa and submucosa layers (Fig. 2C). The level of IL-38 in serum was non-detectable in both DSS-induced colitis and control group (data not shown). Therefore, these findings indicated that the dysregulated expression of IL-38 in murine intestine may be associated with DSS-induced colitis.

3.3. Distribution of IL-38 in intestinal mucosa

To further confirm the cellular source of IL-38 in patients with IBD, colon samples from UC and CD patients were double stained with fluorescence-labeled anti-IL-38 antibody and anti-CD68 antibody, anti-CD3 antibody, or anti-CD19 antibody (Fig. 3). Double staining patterns indicated that IL-38$^+$ cells (red fluorescence) were completely identical to a portion of CD19$^+$ B cells (green fluorescence), and CD19/IL-38 double-positive cells were detected as yellow. On the contrary, IL-38$^+$ cells were not found in CD3$^+$ T cells or CD68$^+$ monocytes/macrophages cells. Therefore, B cells, rather than monocytes/macrophages or T cells were identified as mainly cellular source of IL-38 in the inflamed mucosa of IBD patients.

3.4. rIL-38 treatment ameliorates DSS-induced experimental colitis in mice

Though we demonstrated that IL-38 is increased in colitis, the potential effects of IL-38 in the pathogenesis of IBD needs to be explored. To do so, we administrated DSS-induced colitis mice with mouse IL-38 recombinant protein. The detailed process and dosage regimen of DSS and IL-38 recombinant protein were presented in Fig. 4A. All mice were sacrificed on the 8th day, and relevant indicators were then analyzed. The DSS mice administered with rIL-38 showed significantly alleviated weight loss and colon shortening, as well as DAI compared with those treated with PBS (Fig. 4B–E). In addition, H&E staining indicated that the colonic structure was more severely damaged in PBS-treated DSS mice, characterized by infiltration of inflammatory cells in the lamina propria and loss of crypts, whereas rIL-38 administration significantly ameliorated structural damage and colonic inflammation (Fig. 4F). These findings suggest that IL-38 can effectively alleviate intestinal inflammation in DSS-induced colitis mice.

3.5. IL-38 modulates the production of cytokines in mice with DSS-induced colitis

Previous studies have demonstrated that IL-38 reduces the production of several proinflammatory cytokines in vitro and in vivo (19). To assess whether IL-38 ameliorated the inflammation in DSS-induced colitis through regulating cytokine production in colon tissues, we measured several cytokines in colon tissues from all groups with RT-qPCR. As exhibited in Fig. 5, IL-38 downregulated the mRNA levels of IL-1β, TNF-α and IL-10 ($P < 0.05$), while the expression of IL-6, CCL-2 and CCL-7 were unaffected. In addition, IL-38 significantly reduced the mRNA levels of IL-22 and Foxp3 ($P < 0.05$). Other Th17 cell related cytokines such as IL-17 and IL-23 were also relatively lower in IL-38 group compared with DSS group, although there were no statistical differences between the two groups. The mRNA levels of RORγt and TGF-β did not significantly change. These results demonstrated that administration of rIL-38 could suppress several cytokines such as IL-1β

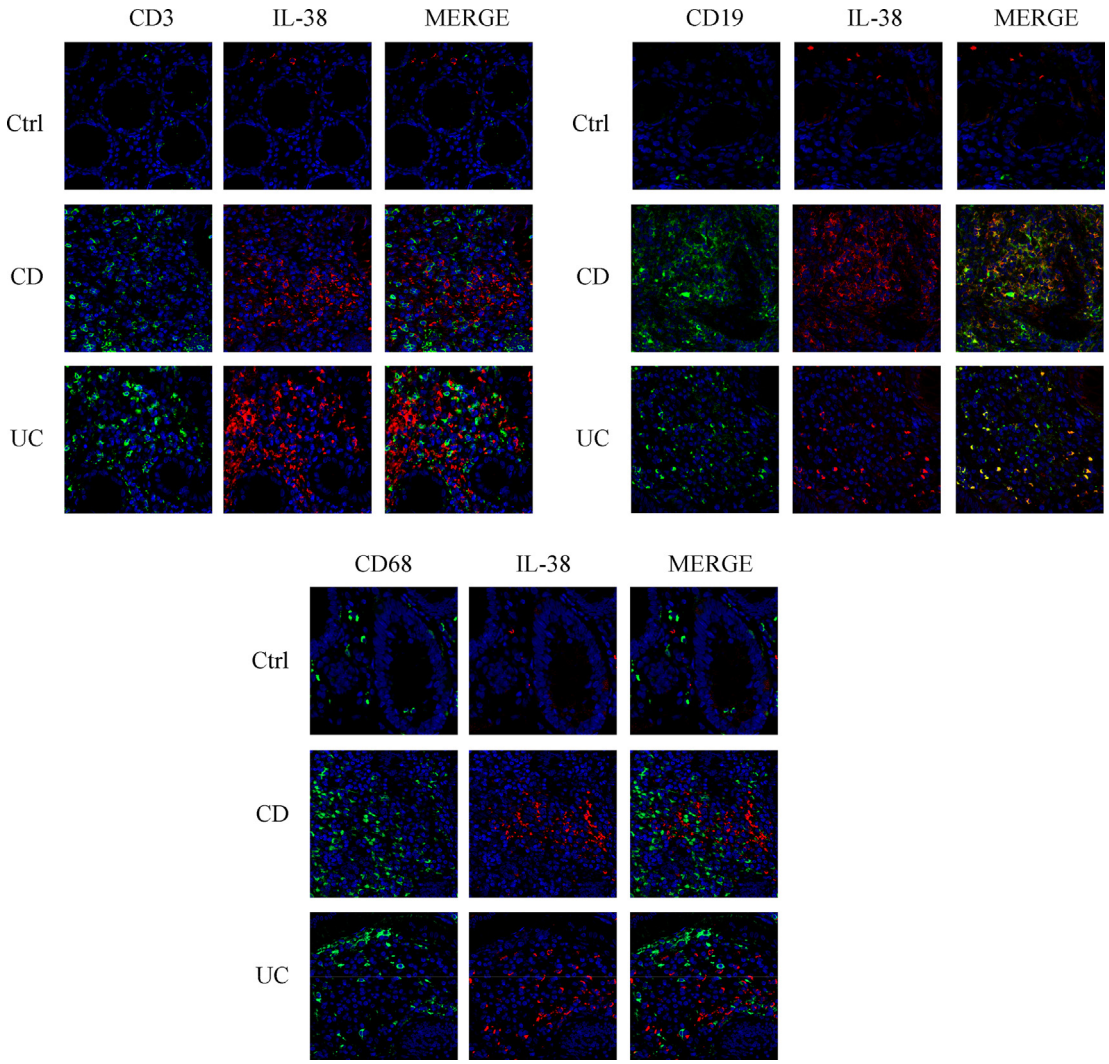

Fig. 3. Distribution of IL-38 in intestinal mucosa. Immunofluorescent staining of cells producing IL-38 in colonic samples from IBD patient. Dual-colored immunofluorescence was used to determine the expression of CD3 (specific for T cell), CD19 (specific for B cell) and CD68 (specific for monocytes/macrophages) (green fluorescence) and the expression of IL-38 (red fluorescence). Double-positive staining were detected by yellow fluorescence in merged panel. Original magnification ×600. Ctrl, control; CD, Crohn's disease; UC, ulcerative colitis.

and TNF-α expression in DSS-induced colitis.

3.6. IL-38 plays an anti-inflammatory role in macrophage

Together, the findings of DSS-induced colitis in mouse models indicated that IL-38 alleviated the inflammation by affecting the production of cytokines. Macrophage plays an important role in mucosal immunity by producing cytokines and chemokines to mediate inflammation. Therefore, we then elucidated whether IL-38 also affected the production of cytokines in RAW 264.7 cells and bone marrow-derived macrophages (BMDM) in vitro. After stimulation with lipopolysaccharide (LPS), rIL-38 treatment significantly decreased the expression of IL-1β, IL-6, TNF-α, IL-10, CCL-2 and CCL-7 in RAW 264.7 cells (Fig. 6A). Similarly, IL-38 intervention in BMDM also strikingly decreased LPS-induced IL-1β, IL-6, TNF-α and CCL-7 mRNA expression, while no differences were observed in IL-10 and CCL-2 (Fig. 6B). Collectively, IL-38 plays a role in triggering an anti-inflammatory effect in LPS-treated RAW 264.7 macrophages and BMDM.

4. Discussion

Inflammatory bowel diseases (IBD), an inflammatory condition of the gastrointestinal tract, are involved in imbalanced cytokines production. In this study, we detected the expression of IL-38 in IBD patients as well as its role in DSS-induced acute mice colitis. Increased expression of IL-38 by infiltrated cells was found in the inflamed mucosa of patients with IBD. Besides, we also showed that IL-38 may suppress intestinal inflammation in mice through inhibiting proinflammatory cytokines production from macrophages.

IL-38 has been reported to be upregulated in some autoimmune diseases. Several studies have reported that IL-38 levels were higher in the synovial membrane of patients with RA than that in healthy controls [15,25,26]. A few preliminary studies have investigated the expression of IL-38 in IBD. Boutet et al showed that IL-38 mRNA levels in inflamed colon lesions were higher than that in non-inflamed colon tissues of CD patients [15]. Moreover, Fonseca-Camarillo et al showed that the percentage of IL-38 immunoreactive cells in active UC and CD patients was increased compared with noninflamed tissues [27]. In this current study, we proved that the mRNA and protein levels of IL-38 in colonic biopsies of both CD and UC patients were higher than controls as well, which is generally consistent with those previous studies. However, the numbers of IBD patients we enrolled were relatively small, especially of CD patients. Meanwhile, we showed that IL-38 increased in the intestine of DSS-induced colitis mice, but not in spleen

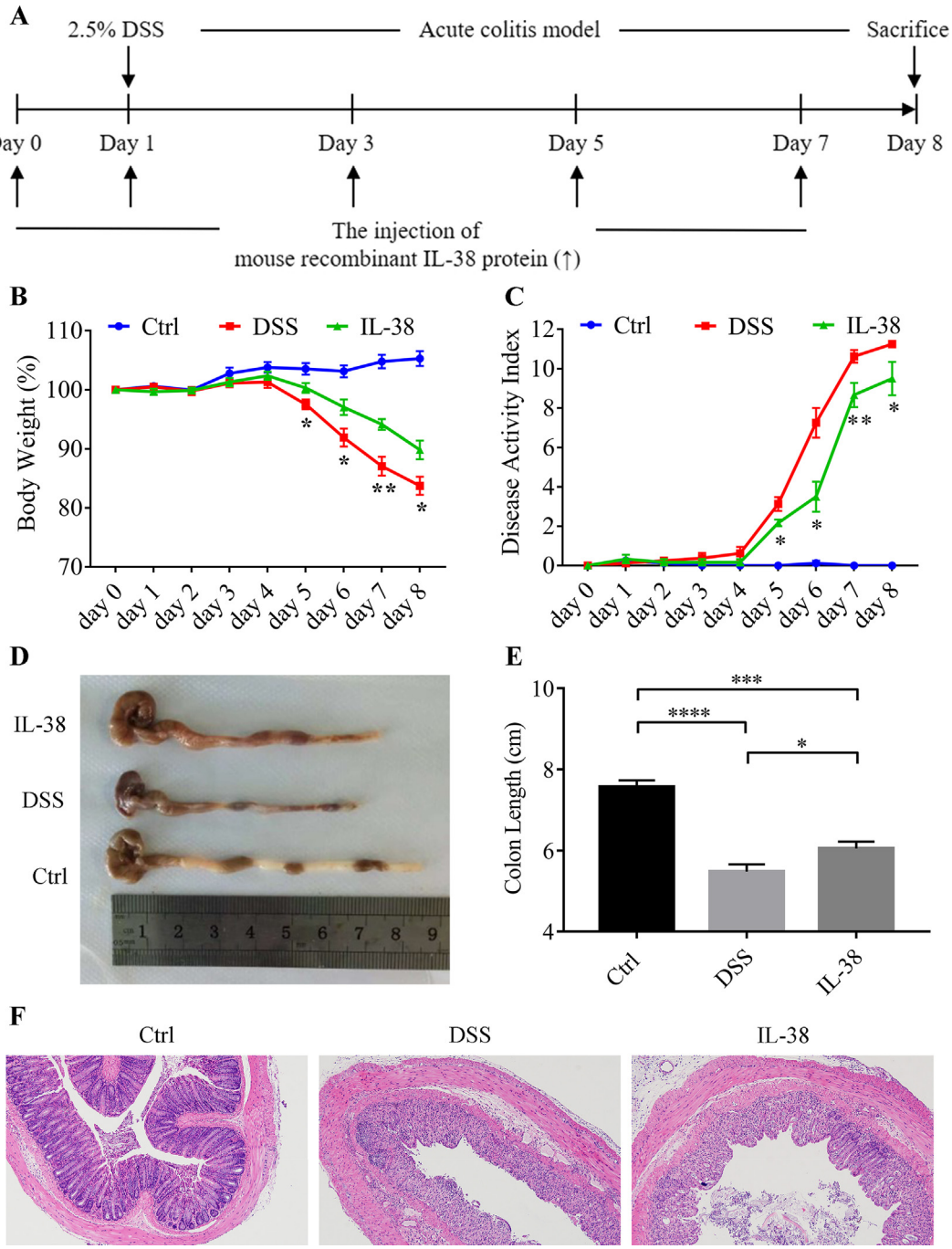

Fig. 4. rIL-38 treatment ameliorates DSS-induced experimental colitis in mice. (A) Detailed process and dosage regimen of DSS and IL-38 recombinant protein. (B) Body weight curve of different groups. (C) Disease activity index was evaluated as the combined score of weight loss, fecal consistency and fecal blood (scored as 0–12). (D) Representative gross photographs of mouse colon and (E) the colon length in different groups were measured. (F) H&E staining of mice colon sections. Original magnification ×100. The data were presented as mean ± SEM. N = 6–8 for each group. Statistical significance was assessed by the Mann–Whitney *U* test. (compared to DSS group,*P < 0.05, ***P < 0.001, ****P < 0.0001).

and mesenteric lymph node.

In addition, for the first time, we assessed the levels of IL-38 in serum from IBD patients and mice colitis. A large proportion of samples were non-detectable because of the low expression of IL-38 in serum, which is similar to the results in SLE. No statistically significant differences in serum IL-38 levels were found. Nevertheless, patients with RA, SLE, myocardial infarction, childhood asthma, and chronic hepatitis B have higher levels of serum IL-38 than healthy control [11,14,25,28–31]. Our data suggests that increased IL-38 is localized in the intestines of IBD patients and DSS mice but not found in the circulation system.

In previous studies, IL-38 protein is expressed by the basal epithelial cell of skin and proliferating B cells of the tonsil, but not by $CD45^+$ T cells or $CD14^+$ monocytes of tonsil [8]. Beyond that, IL-38 is also produced by keratinocytes and synovial fibroblast from patients with RA [13]. However, the cellular source of IL-38 in the intestinal tract remains unknown. Therefore, we carried out immunofluorescent double-staining to explore the cellular source of IL-38 in inflamed colonic mucosa. According to our results, B cells were identified as the main source of IL-38 in the inflamed mucosa of IBD. Nonetheless, IL-

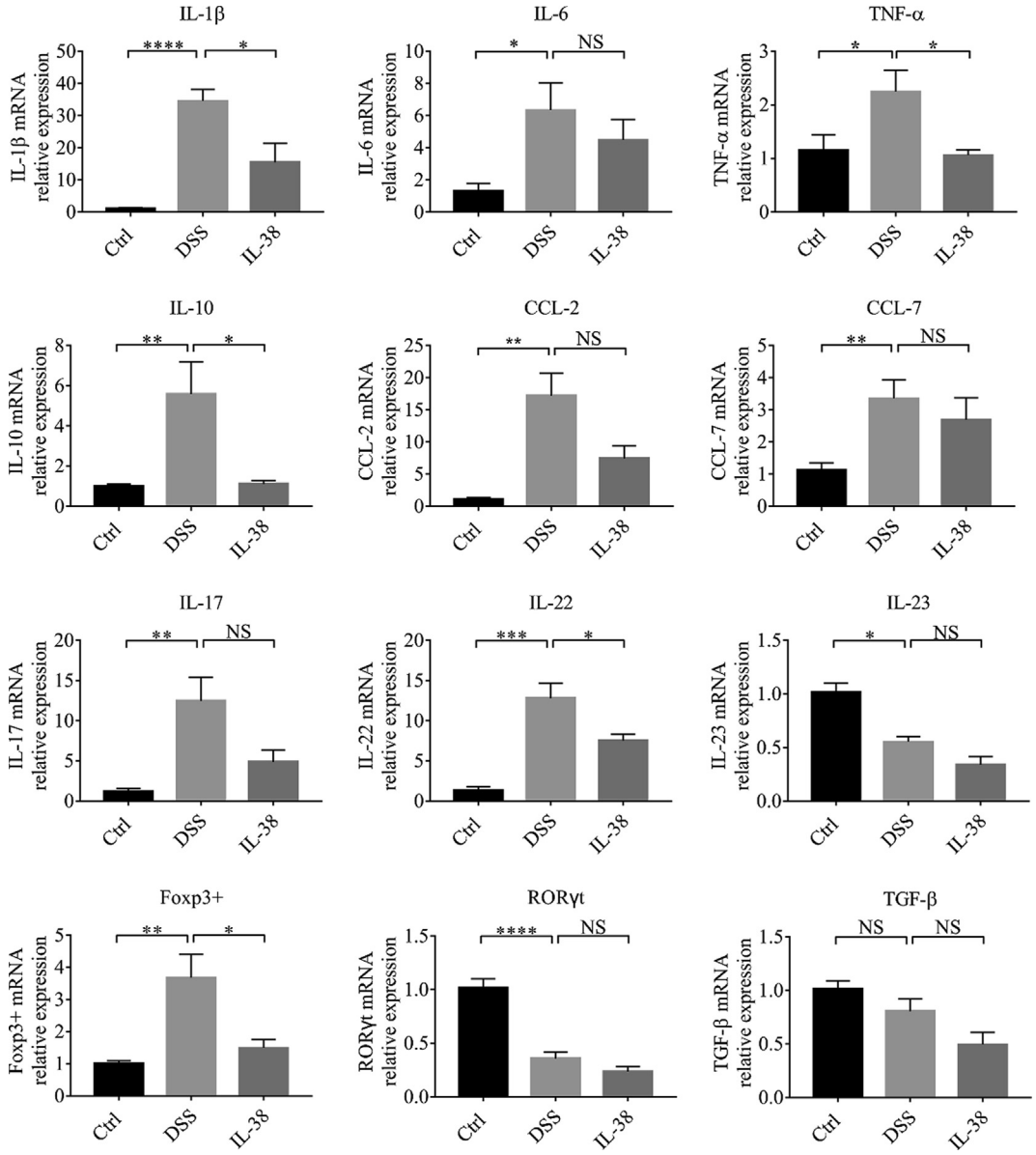

Fig. 5. IL-38 modulates the production of cytokines in mice with DSS-induced colitis. Total mRNA was extracted from colonic samples to determine the mRNA expression of IL-1β, IL-6, TNF-α, CCL-2, CCL-7, IL-10, IL-17, IL-22, IL-23, Foxp3+, RORγt and TGF-β by RT-qPCR. The data are shown as mean ± SEM, N = 6–8/group. Statistical significance was assessed by the unpaired, two-tailed Student's *t*-test. NS, P > 0.05; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

38^+ cells were not detected in $CD68^+$ monocytes/macrophages and $CD3^+$ T cells. A recent study indicated that IL-38 was expressed by some $CD123^-$ perivascular cells of colon tissues in IBD patients [27], which was also observed in our study. In summary, IL-38 expression was locally increased in the intestinal mucosa mainly produced by B cells.

Owing to its homology with IL-1F inhibitors such as IL-1Ra and IL-36Ra, it is believed that IL-38 plays an anti-inflammatory role in several autoimmune diseases. For instance, $IL\text{-}38^{-/-}$ mice exhibited more severe K/BxN serum transfer arthritis (STIA), which was linked to the higher expression of IL-1β and IL-6 in joints [26]. Local IL-38 overexpression induced by adeno-associated virus was associated with the decreased clinical score of collagen-induced arthritis (CIA) and STIA by suppressing macrophage infiltration, as well as expression of Th17 cytokines (IL-17, IL-22 and IL-23), TNF-α and RANKL [13]. In SLE spontaneous murine model (MRL/lpr mice), the administration of IL-38 recombinant protein improved clinical symptoms such as skin injury and proteinuria through reducing serum IL-17 and IL-22 whereas the mRNA expression of Th1 and Th2 cytokine remained unchanged [32]. In liver injury mice model induced concanavalin A, IL-38 overexpression resulted in reduced hepatic toxicity and decreased serum levels of IL-6, TNF-α, INF-γ, IL-17 and IL-22, but not of IL-10 [29].

Although IL-38 has been proved to exert anti-inflammatory effects and also decreases Th17 cytokines expressions in several diseases, the exact functions of IL-38 in IBD are still unknown. In this study, therefore, we firstly investigated the role of IL-38 in DSS-induced acute colitis mice. As shown in results, rIL-38 alleviated weight loss, colon length shortening, as well as reduced DAI score, and improved structural damage and colonic inflammation in H&E staining of DSS colitis mice. Thus we have shown that IL-38 can effectively alleviate intestinal inflammation in DSS-induced acute colitis. We further demonstrated that IL-38 treatment reduced IL-1β, TNF-α and CCL-2 expression, but the changes of Th17 cell-associated cytokines was not obvious.

To our knowledge, macrophages play a vital role in regulating mucosal immune by producing cytokines and chemokines. Therefore, for the first time, we elucidated the biological role of IL-38 in murine

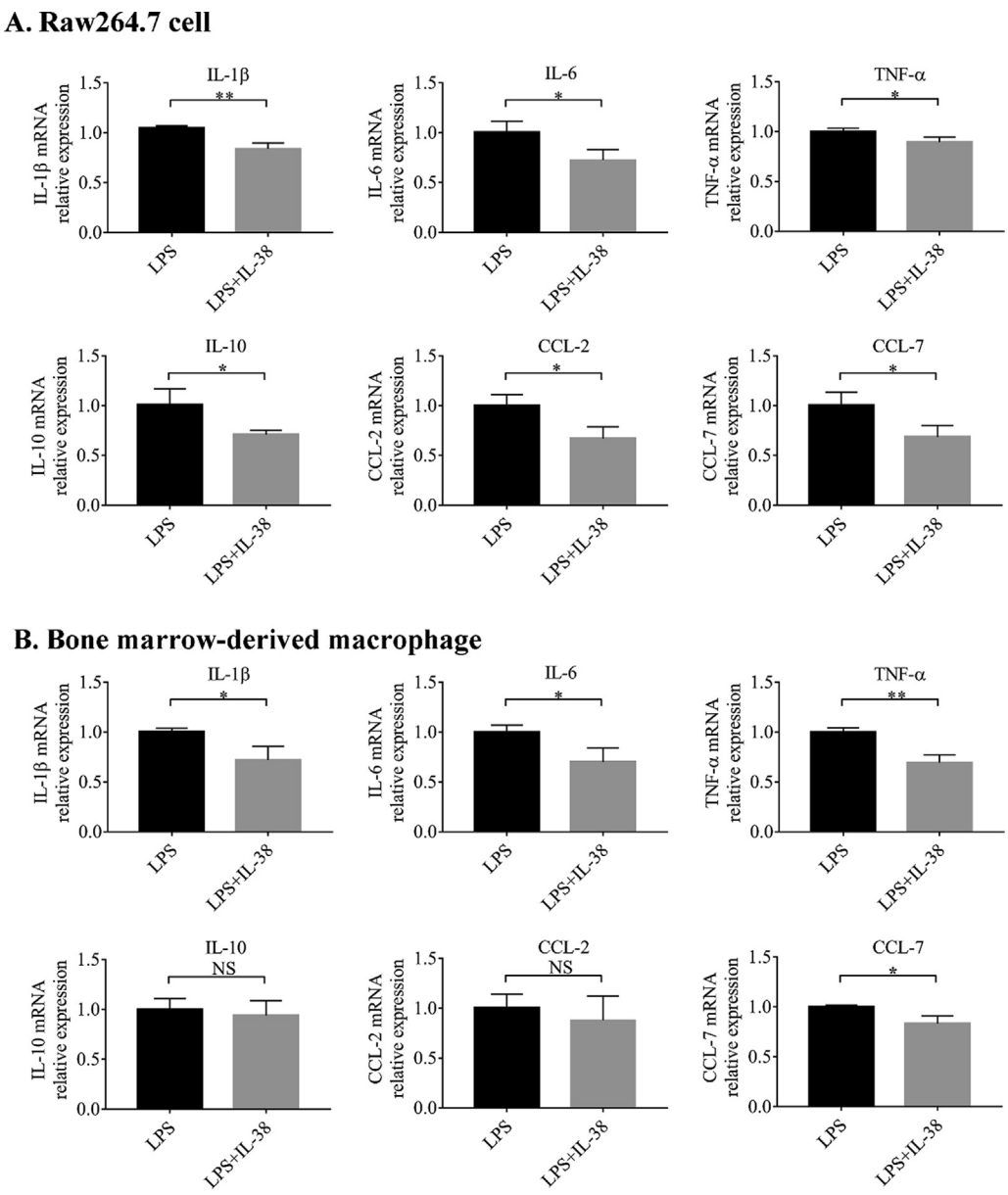

Fig. 6. IL-38 plays an anti-inflammatory role in RAW 264.7 cell and BMDM. (A) RAW 264.7 cells were intervened with mouse IL-38 recombinant protein at 100 ng/ml in the presence of LPS 100 ng/ml for 12 h. The mRNA level of IL-1β, IL-6, TNF-α, IL-10, CCL-2 and CCL-7 was measured by RT-qPCR. (B) Bone Marrow Derived Macrophages were obtained and were treated with mouse IL-38 recombinant protein at 100 ng/ml in the presence of LPS 100 ng/ml for 12 h. The mRNA level of IL-1β, IL-6, TNF-α, IL-10, CCL-2 and CCL-7 was measured by RT-qPCR. Data are presented as means ± SEM. Statistical significance was assessed the unpaired, two-tailed Student's *t*-test. NS, P > 0.05; *P < 0.05; **P < 0.01.

macrophage cell (RAW 264.7 cells and BMDM). Our study found that rIL-38 decreased the expression of IL-1β, IL-6, TNF-α, IL-10, CCL-2 and CCL-7 in LPS-stimulated RAW 264.7 cells significantly as well as IL-1β, IL-6, TNF-α and CCL-7 mRNA in LPS-stimulated BMDM. These findings are comparable to the results of previous studies in human. Dinarello et al demonstrated that IL-38 can restrain the expression of Th17 related cytokines of PBMC from healthy donors stimulated by *C. albicans* [10]. Nold et al also found that down-regulation of endogenous IL-38 in PBMC increased the production of pro-inflammatory cytokines such as IL-6, APRIL and CCL-2 in response to TLR ligands [11]. Mora et al revealed that IL-38 inhibited LPS-stimulated IL-6 and IL-8 production by macrophages and IL-38-treated macrophages in conditioned media inhibited IL-17 production by $CD3^+/CD4^+$ T cells from healthy donors [12]. After stimulated with LPS, THP-1 cells produced lower levels of IL-1β, TNF-α and IL-17 in the presence of IL-38 recombinant protein [33]. Collectively, IL-38 exerts an anti-inflammatory effect in LPS-treated RAW 264.7 macrophages and BMDM, which may be one possible mechanism of suppressing intestinal inflammation in DSS mice.

5. Conclusions

In the present study, we clarified that IL-38 is increased in the intestine but not in the circulation system of IBD patients and DSS-colitis mice model. We showed that IL-38 is mainly derived from B cells. IL-38 can alleviate intestinal inflammation in DSS-induced acute colitis by inhibiting the release of inflammatory mediators probably from macrophages. However, further studies are demanded to ascertain the precise mechanism by which IL-38 attenuates the intestinal inflammation, the receptors and downstream signaling pathway of IL-38. Therefore, our study may provide new insights and therapeutic targets for treating IBD.

6. Authors' contributions

Cheng Xie: the conception and design of the study, data collection, data analysis, and drafting the paper. Wei Yan: the conception and design of the study, patient recruitment, and critical revision of the manuscript. Runze Quan: data collection and critical revision of the manuscript. Chaoyue Chen: data collection and critical revision of the manuscript. Lei Tu: patient recruitment, and critical revision of the

manuscript. Xiaohua Hou: study design, patient recruitment, drafting the paper, and critical revision of the manuscript. Yu Fu: study design, patient recruitment, data analysis, interpretation of data and critical revision of the manuscript. All authors read and approved the final version of the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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