表2　動脈硬化性疾患予防ガイドライン2007年版
—患者カテゴリー別管理目標値—

治療方針の原則	カテゴリー	LDL-C以外の主要危険因子*	脂質管理目標値(mg/dL) LDL-C	HDL-C	TG
一次予防 まず生活習慣の改善を行った後，薬物治療の適応を考慮する．	Ⅰ (低リスク群)	0	<160		
	Ⅱ (中リスク群)	1～2	<140		
	Ⅲ (高リスク群)	3以上	<120	≧40	<150
二次予防 生活習慣の改善とともに薬物治療を考慮する．	冠動脈疾患の既往		<100		

脂質管理と同時にほかの危険因子(喫煙，高血圧や糖尿病の治療など)を是正する必要がある．
*LDL-C値以外の主要危険因子
加齢(男性≧45歳，女性≧55歳)，高血圧，糖尿病(耐糖能異常を含む)，喫煙，冠動脈疾患の家族歴，低HDL-C血症(<40 mg/dL)
・糖尿病，脳梗塞，閉塞性動脈硬化症の合併はカテゴリーⅢとする．
・家族性高コレステロール血症については別章を参照のこと．
(日本動脈硬化学会：動脈硬化性疾患予防ガイドライン2007)

2)に示された脂質管理目標値を，成人と同様達成することが妥当と考えられる．特に，成人期から脂質異常症そのほかの危険因子の合併によって本ガイドラインの基準に従い脂質管理を受けていた患者の治療が，高齢であるという理由で中断されることがあってはならない．

一方で，75歳以上の後期高齢者においては，動脈硬化性疾患の危険因子としての高LDL-C血症の意義づけや，スタチンなどによる脂質低下療法の有効性が十分明らかではない．欧米でも，このような高齢者に対する脂質管理の数値目標は示されていない．したがって，「動脈硬化性疾患予防ガイドライン」では，個々の患者の病態やADLを考慮し，後期高齢者の脂質異常症治療については主治医の判断に委ねられている．

実際の高齢者脂質異常症の治療においては，動脈硬化性疾患以外の合併症や多くの併用薬の存在，臓器予備能や薬物代謝能の低下など留意すべき点が多い．高齢者においても，脂質異常症の治療の基本は食事療法，運動療法であり，安易な薬物治療は避けるべきである．一方，厳格な食事療法による栄養状態の悪化や，過度の運動による腰背痛や膝関節痛の発症，これらによるADLの低下が起こり得る．薬物の使用に際しては，副作用の出現に常に留意する必要がある．このように，高齢者の脂質異常症治療に際しては，個々の患者の病態や背景に配慮し細心の注意を払い，柔軟に対応することが求められる．

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SR-PSOX/CXCL16 plays a critical role in the progression of colonic inflammation

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ABSTRACT

Background and aims Inflammatory bowel disease (IBD) is initiated and perpetuated by a dysregulated immune response to unknown environmental antigens such as luminal bacteria in genetically susceptible hosts. SR-PSOX/CXCL16, a scavenger receptor that binds phosphatidylserine and oxidised lipoprotein, has both phagocytic activity and chemotactic properties. The aim of this study was to investigate the role of SR-PSOX/CXCL16 in patients with IBD and experimental murine colitis.

Methods The serum levels of SR-PSOX/CXCL16 were measured in patients with IBD. The roles of SR-PSOX/CXCL16 in phagocytosis of bacterial components and cytokine production by macrophages from wild-type (WT) and SR-PSOX/CXCL16 knockout (KO) mice were assessed. Colitis was induced by administering dextran sulfate sodium (DSS) to WT and SR-PSOX/CXCL16 KO mice. Colonic inflammation was analysed by clinical, histological and immunological parameters. Finally, the effect of a monoclonal antibody (mAb) to SR-PSOX/CXCL16 on DSS-induced colitis and trinitrobenzene sulfonic acid-induced colitis models was evaluated.

Results Serum levels of SR-PSOX/CXCL16 correlated significantly with the disease activity of patients with IBD. Ex vivo experiments showed that SR-PSOX/CXCL16 was involved in both phagocytosis of bacterial antigens and the T helper 1 immune response through the production of interleukin 12 and interferon γ. In vivo murine experiments demonstrated the upregulated gene expression of SR-PSOX/CXCL16 in inflamed colonic tissues and the predominant expression of SR-PSOX/CXCL16 on macrophages. SR-PSOX/CXCL16 KO mice were less susceptible to colonic inflammation than were their WT littermates. Administration of SR-PSOX/CXCL16 mAb ameliorated the condition in the two different experimental colitis models.

Conclusions SR-PSOX/CXCL16 plays a critical role in colonic inflammation and could be a potential therapeutic target for patients with IBD.

Significance of this study

What is already known about this subject?

- Inflammatory bowel disease (IBD) is associated with a dysregulated immune response to unknown environmental antigens including luminal bacteria in genetically susceptible hosts.
- SR-PSOX/CXCL16 is associated with several inflammatory diseases.
- SR-PSOX/CXCL16 is constitutively expressed in the small intestine but not in normal colonic tissues.
- SR-PSOX/CXCL16 has two different biological activities: as a scavenger receptor and as a chemokine.

What are the new findings?

- Serum levels of SR-PSOX/CXCL16 correlated with the disease activity of IBD.
- SR-PSOX/CXCL16 expression is upregulated in inflamed colonic tissues and predominantly detected on macrophages.
- SR-PSOX/CXCL16 is involved in not only phagocytosis of bacterial antigens but also the T helper 1 immune response in the progression of colitis.
- SR-PSOX/CXCL16 monoclonal antibody ameliorated the condition in two different experimental colitis models.

How might it impact on clinical practice in the foreseeable future?

- Our data provide the first evidence for a critical role for SR-PSOX/CXCL16 in the progression of colonic inflammation ex vivo and in vivo. These data suggest that SR-PSOX/CXCL16 could be a potential therapeutic target for patients with IBD.

INTRODUCTION

Inflammatory bowel diseases (IBDs), including Crohn's disease (CD) and ulcerative colitis (UC), are chronic and relapsing-remitting conditions with unknown aetiology. Previous clinical and basic observations suggest that inflammation is initiated and perpetuated by a dysregulated immune response to unknown environmental antigens such as luminal bacteria in genetically susceptible hosts.[1] Recent genome-wide association studies showed that genes involved in both innate and adaptive immune responses could be risk factors for developing IBD.[2 3] Moreover, an abnormal response of intestinal macrophages to commensal bacteria was reported to result in chronic intestinal inflammation.[4] Therefore, it is important to investigate the relationship between luminal bacteria and antigen-presenting cells (APCs) in the pathogenesis of IBD. Indeed, we showed that macrophage-targeting treatment ameliorates colonic inflammation in an experimental colitis model.[5] Thus, the control of

molecules related to macrophages appears to be a promising approach for the treatment of IBD.

Chemokines are a superfamily of small chemotactic cytokines and are classified into four major subfamilies on the basis of the motif of the first two cysteine residues: CC, CXC, C and CX3C subfamilies.[6] The most important function of chemokines is the ability to regulate leucocyte trafficking and retention in lymphoid tissues and in peripheral tissues in both homeostasis and inflammation.[7 8] The expression of several chemokines increases in the colonic tissues of both experimental murine colitis models and patients with IBD,[9 10] and these chemokines are suggested to play a role in the pathophysiology of colitis.

SR-PSOX/CXCL16, a scavenger receptor that binds phosphatidylserine and oxidised lipoprotein, is a chemokine of the CXC family and has been identified as a novel transmembrane protein.[11–13] In the static state, SR-PSOX/CXCL16 is expressed in various lymphoid tissues including the thymus, spleen, lymph nodes and Peyer's patches, and in non-lymphoid tissues including the lung, liver, kidney and small intestine, but not colonic tissue.[11 12] Among immune cells, SR-PSOX/CXCL16 is found primarily on the surface of APCs such as monocytes/macrophages and dendritic cells.[11–13] SR-PSOX/CXCL16 has two different biological activities: as a scavenger receptor that mediates adhesion and phagocytosis of both Gram-positive and Gram-negative bacteria by APCs[14] and as a chemokine for CXCR6-expressing cells such as naïve CD8 T cells, T helper 1 (Th1)-polarised CD4 and CD8 T cells, and natural killer T cells. Accordingly, it is possible that SR-PSOX/CXCL16 plays an important role in both innate and adaptive immunity by mediating the uptake of bacteria as well as the recruitment of CXCR6-expressing T cells by APCs. Although SR-PSOX/CXCL16 has been shown to be involved in several inflammatory conditions,[15–17] little is known about the function of SR-PSOX/CXCL16 in intestinal inflammation.

The aim of this study was to elucidate the role of SR-PSOX/CXCL16 in the pathophysiology of IBD. We first measured the serum levels of SR-PSOX/CXCL16 in patients with IBD. We assessed the role of SR-PSOX/CXCL16 in phagocytosis of bacterial components and cytokine production by macrophages in SR-PSOX/CXCL16 knockout (KO) mice. Next, we investigated the role of SR-PSOX/CXCL16 in a dextran sulfate sodium (DSS)-induced colitis model. Finally, we examined the effects of a monoclonal antibody (mAb) to SR-PSOX/CXCL16 in two experimental murine colitis models: DSS-induced colitis and 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis.

MATERIALS AND METHODS

Human serum samples

Human serum samples were obtained from 14 patients (11 men and 3 women; mean age, 27.8±6.4 years) with active CD, 16 patients (9 men and 7 women; 27.7±7.4 years) with inactive CD, 16 patients (10 men and 6 women; 27.2±15.6 years) with active UC, 13 patients (10 men and 3 women; 29.5±14.8 years) with inactive UC and 16 healthy volunteers (15 men and 1 woman; 34.6±3.1 years). The clinical characteristics of patients with CD and UC are shown in table 1. The disease activity of the patients with CD and UC was determined according to the Crohn's Disease Activity Index (CDAI)[18] and the Clinical Activity Index (CAI),[19] respectively. A CDAI ≥150 and a CAI ≥5 was defined as active CD and active UC, respectively.[20 21] Informed consent was obtained from all patients and volunteers, and the experimental design using these samples was approved by the Kyoto University Hospital Ethics Committee.

Table 1 Clinical characteristics of patients

(A) Patients with CD	Active CD (n=14)	Inactive CD (n=16)
Age (years)	27.8±6.4	27.7±7.4
Gender (M/F)	11/3	9/7
Disease duration (years)	8.0±7.3	8.3±7.3
Disease location (n)	Ileal only (3)	Ileal only (8)
	Ileocolonic (7)	Ileocolonic (4)
	Colonic only (4)	Colonic only (4)
CDAI	248.0±87	110.1±25.8*
(B) Patients with UC	**Active UC (n=16)**	**Inactive UC (n=13)**
Age (years)	27.2±15.6	29.5±14.8
Gender (M/F)	10/6	10/3
Disease duration (years)	2.1±1.8	9.1±4.8*
Disease location (n)	Pancolitis (9)	Pancolitis (7)
	Left-sided colitis (7)	Left-sided colitis (5)
	Proctitis (0)	Proctitis (1)
CAI	10.9±2.9	1.2±1.2*

The values are expressed as mean±SD or number of patients.
*$p<0.01$ between active and inactive CD or UC.
CAI, Clinical Activity Index; CD, Crohn's disease; CDAI, Crohn's Disease Activity Index; F, female; M, male, UC, ulcerative colitis.

Mice

SR-PSOX/CXCL16 KO mice were generated in collaboration with Sankyo Co. (Tokyo, Japan) as described previously.[22] Heterozygous mice were generated by crossing SR-PSOX/CXCL16 KO mice and C57BL/6 mice, and were intercrossed to obtain homozygous SR-PSOX/CXCL16 KO and wild-type (WT) littermates. The genotyping of F_2 mice was performed by PCR at least twice using the following primers: 5′-TACCGCAGGGTACTTTGGATCA-3′ and 5′-TTGCGCTCAAAGCAGTCCACTA-3′ for detection of the WT SR-PSOX/CXCL16 allele (351 bp), and 5′-GGATCTCCTGTCATCTCACCTTGC-3′ and 5′-CGGCCACAGTCGATGAATCCAGAA-3′ for detection of the KO allele (333 bp). SR-PSOX/CXCL16 KO mice and their WT littermates from intercrosses of heterozygous mice were used in the experiments. C57BL/6 mice and SJL/J mice were purchased from Japan SLC (Shizuoka, Japan) and Charles River Japan (Kanagawa, Japan), respectively. All mice were fed with standard laboratory chow and water ad libitum, and housed in specific pathogen-free conditions in the animal facility of Kyoto University. All experiments were performed with female mice at 8–12 weeks of age according to the protocol approved by the Animal Protection Committee of our institution.

Preparation of thioglycollate-elicited peritoneal macrophages

SR-PSOX/CXCL16 KO and WT mice were injected intraperitoneally with 3 ml of 3% thioglycollate (Eiken Chemical Co., Tokyo, Japan) and peritoneal exudate cells (PECs) were harvested 4 days later. Cells were cultured with complete RPMI medium (RPMI 1640 medium (Gibco BRL, Eggenstein, Germany) supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/ml streptomycin (Sigma Chemical Co., St Louis, Missouri, USA) and 100 μg/ml penicillin (Sigma Chemical Co.)) for 2 h and, after removal of non-adherent cells, adherent PECs were cultured as peritoneal macrophages. Adherent PECs were resuspended and adjusted to a concentration of 1×10^6 cells/ml.

Preparation of caecal bacterial lysates (CBLs)

CBLs were prepared directly from the caecal contents of SR-PSOX/CXCL16 KO and WT mice according to a protocol described by Cong *et al*.[23] The sterility of the lysates was confirmed by culture.

Phagocytosis assay

The ex vivo phagocytosis assay was performed using pHrodo *Escherichia coli* BioParticles conjugate for phagocytosis (Invitrogen) according to the manufacturer's instructions. The fluorescence intensity was measured using a microplate reader (Fluoroskan Ascent FL: Labsystems, Helsinki, Finland) at the indicated times. For fluorescence microscopic observation of phagocytosis, peritoneal macrophages were seeded at 3×10^5 cells/well in 8-well Lab-Tek chamber glass slides (NUNC Roskilde, Denmark) and incubated overnight in complete medium. The wells were washed with phosphate-buffered saline (PBS), fluorescent particles were added and the slides were observed using fluorescence microscopy (Olympus, Tokyo, Japan) at the indicated times.

Stimulation and cytokine production assay of peritoneal macrophages

Peritoneal macrophages were seeded at 2.5×10^5 cells/well in 48-well culture plates and incubated overnight in complete medium. Cells were primed with 500 U/ml interferon γ (IFNγ; R&D Systems, Minneapolis, Minnesota, USA) for 16 h and then stimulated with 100 ng/ml lipopolysaccharide (LPS (L5668-2ML); Sigma Chemical Co.) or 30 μg/ml CBL for 24 h. The supernatants were collected and subjected to analysis of cytokine production by ELISA.

Induction of experimental colitis

DSS-colitis was induced in SR-PSOX/CXCL16 KO mice, WT littermates and C57BL/6 mice using a modification of the method described by Inoue *et al*.[24] In brief, to induce colitis, 3% DSS (molecular mass, 36–50 kDa; MP Biomedicals, Solon, Ohio, USA) in regular drinking water was administered for 5 days (from day 0 to 4), and then regular drinking water was given from day 5. Normal control mice received regular drinking water throughout the experiment. The mice were sacrificed on day 8 or day 14 to evaluate the acute inflammatory phase or the restitution phase, respectively. TNBS-colitis was induced in SJL/J mice using a modification of the method described by Neurath *et al*.[25]

The neutralising effect of mAb to SR-PSOX/CXCL16 on colitis models

To investigate the effect of blocking SR-PSOX/CXCL16 on experimental colitis, C57BL/6 mice with DSS-induced colitis and SJL/J mice with TNBS-induced colitis were injected intraperitoneally with 500 μg of mAb to SR-PSOX/CXCL16[26] dissolved in 200 μl of PBS or an equal amount of control rat immunoglobulin G (IgG) (MP Biomedicals) in PBS once a day from days 1 to 7 and days 1 to 3, respectively.

Microscopic assessment of colitis

The colonic tissues were treated using the same method as in Matsuura *et al*[27] and then analysed histologically in a blind manner. Histological damage of DSS- and TNBS-induced colitis was quantified using the histological scoring system described by Williams *et al*[28] and Elson *et al*,[29] respectively.

Colon fragment culture

Fragment culture of distal colon segments was performed according to the published method.[30] Culture supernatants were collected and stored at −80°C until assayed.

Isolation and stimulation of mesenteric lymph node (MLN) cells

MLNs were isolated as described previously.[31] MLN cells (2×10^5 cells/well) were incubated with immobilised anti-CD3 (5 μg/ml antimouse CD3ε; BD Pharmingen, San Diego, California, USA) plus CD28 (2 μg/ml antimouse CD28, BD Pharmingen) in 200 μl of complete medium containing 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co.) in a 5% CO_2 incubator at 37°C for 72 h. The supernatant of the culture medium was collected and stored at −80°C until assayed.

Isolation of colonic lamina propria macrophages

Lamina propria macrophages were isolated using a modified protocol as described previously.[4] Briefly, mice were sacrificed, and colonic tissues were removed, washed with cold PBS and cut into three pieces. The resected colonic tissues were shaken with Hanks' balanced salt solution (HBSS; Gibco) for 1 min at 2800 rpm in a Mini Bead Beater (Biospec Products, Bartlesville, Oklahoma, USA) to remove faeces and mucus, dissected into small pieces and then incubated with HBSS containing 5% fetal calf serum and 5 mM EDTA (Gibco) for 30 min at 37°C under rotation at 120 rpm. After washing, the pieces were incubated with complete RPMI medium containing 1 mg/ml collagenase type II (Invitrogen, Carlsbad, California, USA), 1 mg/ml dispase (Gibco) and 40 μg/ml DNase (Roche, Mannheim, Germany) for 60 min at 37°C under rotation at 120 rpm to digest colonic tissues. After washing, the extracted cells were filtered and subjected to a magnetic cell separation system (Miltenyi Biotec, Auburn, California, USA) with antimouse CD11b microbeads to separate colonic lamina propria macrophages. Cell viability was determined by trypan blue staining, and >95% purity was confirmed by flow cytometry.

ELISA

The serum level of SR-PSOX/CXCL16 in the human subjects was determined quantitatively using a human CXCL16 immunoassay ELISA kit (R&D Systems). In the mouse model, the levels of SR-PSOX/CXCL16 in the serum and the supernatant of the colon fragment culture were measured using a mouse CXCL16 ELISA kit (R&D Systems). The cytokine levels of interleukin 6 (IL-6) and IL-12/23 p40 in the supernatants of the culture medium of thioglycollate-elicited peritoneal macrophage were measured using mouse ELISA kits (eBioscience, San Diego, California, USA). The secretions of IFNγ and IL-17 into the supernatants of culture medium in activated MLN cells were determined by mouse ELISA kits (eBioscience).

Quantitative analysis of gene expression of SR-PSOX/CXCL16 in colonic tissue

mRNA was assessed using colonic tissues isolated from the distal colon of WT mice with or without DSS-induced colitis. The extraction of total RNA, generation of cDNA and real-time reverse transcription–PCR (RT–PCR) were performed as described previously.[31] The following primers were used: SR-PSOX/CXCL16, 5′-GGCTTTGGACCCTTGTCTCTTG-3′ (forward) and 5′-TTGCGCTCAAAGCAGTCCACT-3′ (reverse); and glyceraldehyde phosphate dehydrogenase (GAPDH), 5′-CAA CTTTGTCAAGCTCATTTCC-3′ (forward) and 5′-GGTCCAG GGTTTCTTACTCC-3′ (reverse).

Western blot analysis

Colonic tissues were lysed in RIPA buffer (1% Triton X-100, 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 20 mmol/l Tris–HCl (pH 7.4)) with protease inhibitor cocktail (Sigma Chemical Co.), and the insoluble material was removed by centrifugation at 12 000 *g* for 5 min at 4°C. The supernatants were boiled in sample buffer (0.05 mol/l Tris–HCl, 2% SDS, 6% β-mercaptoethanol, 10% glycerol, 1.25% bromophenol blue),

subjected to SDS–PAGE (10% polyacrylamide gels) and transferred onto polyvinylidine fluoride membranes (PALL Corporation, Pensacola, Florida, USA). The membranes were blocked with blocking buffer (Tris-buffered saline with 0.5% Tween-20 (TBS-T) containing 5% milk powder) and then incubated with a 1:1000 dilution of anti-CXCL16 mAb and with 1:5000 dilution

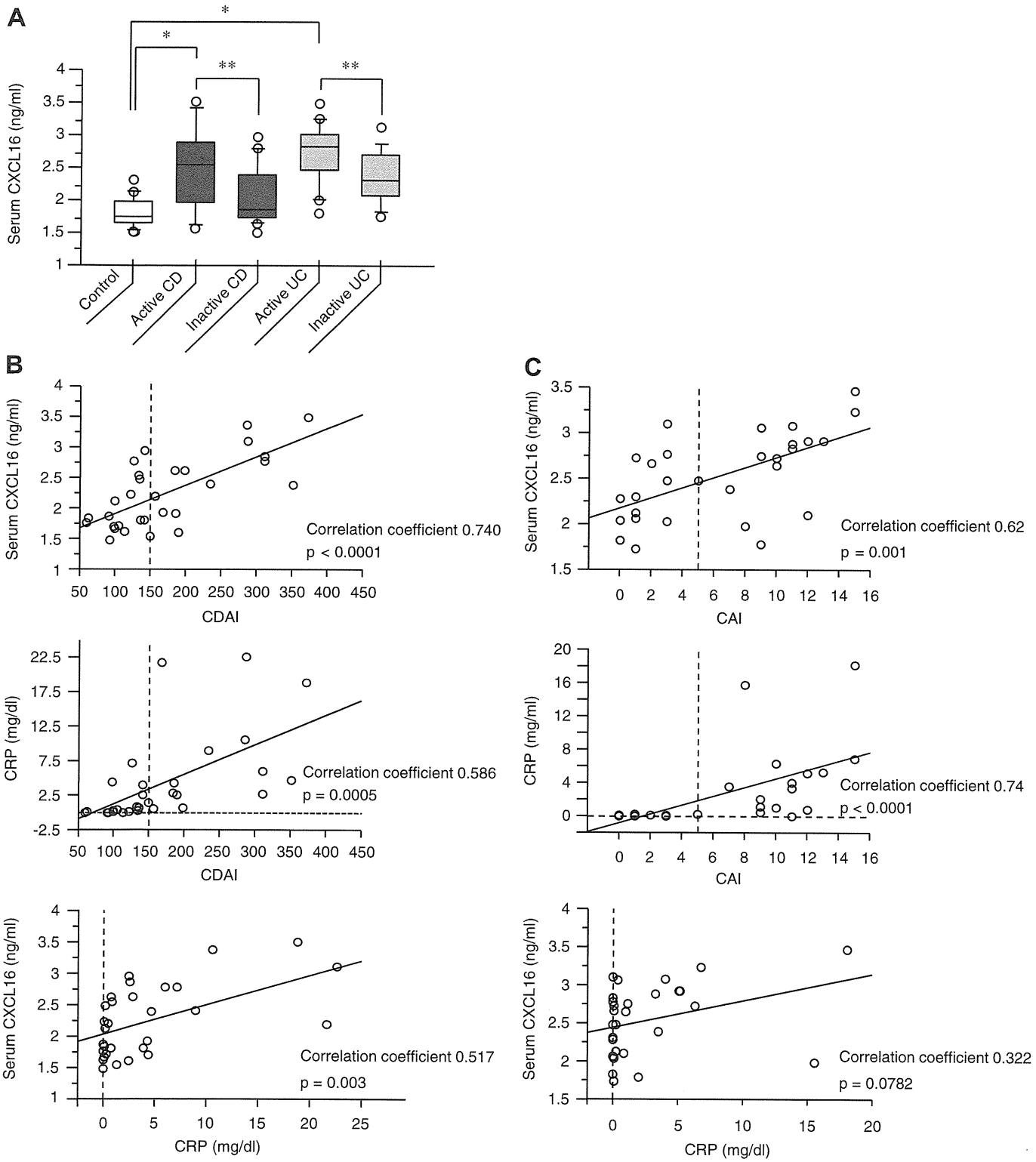

Figure 1 Serum levels of human SR-PSOX/CXCL16 are higher in patients with active inflammatory bowel disease (IBD). Serum samples were obtained from patients with active Crohn's diease (CD) (n=14), inactive CD (n=16), active ulcerative colitis (UC) (n=16) and inactive UC (n=13), and from healthy controls (n=16). Results are expressed as means±SEM. *$p<0.05$ compared with control and **$p<0.05$ between patients with active IBD and inactive IBD. (A) The statistical difference of serum SR-PSOX/CXCL16 in patients with IBD and healthy controls was determined by unpaired Student t test. (B) The relationship among serum SR-PSOX/CXCL16, C-reactive protein (CRP) and Crohn's Disease Activity Index (CDAI) in patients with CD was assessed by the Pearson correlation coefficient test. (C) The relationship among serum SR-PSOX/CXCL16, CRP and Clinical Activity Index (CAI) in patients with UC was investigated by the Pearson correlation coefficient test or Spearman correlation test.

of anti-β-actin mAb (Sigma Chemical Co.) overnight at 4°C. The membranes were washed and incubated with a horseradish peroxidase (HRP)-conjugated IgG. The immunoreactive bands were visualised with Immobilon Western chemiluminescent HRP substrate (Millipore, Billerica, Massachusetts, USA), and the images were recorded using a chemiluminescent image reader (LAS-3000; Fujifilm, Tokyo, Japan).

Immunohistochemistry

For SR-PSOX/CXCL16 immunostaining, colonic tissues of WT mice with or without DSS-induced colitis, and Peyer's patches as the positive control, were prepared as described previously.[24] The sections were incubated with biotinylated anti-CXCL16 antibody (1:250; R&D Systems) or goat IgG isotype control overnight at 4°C. After washing, the sections were incubated with

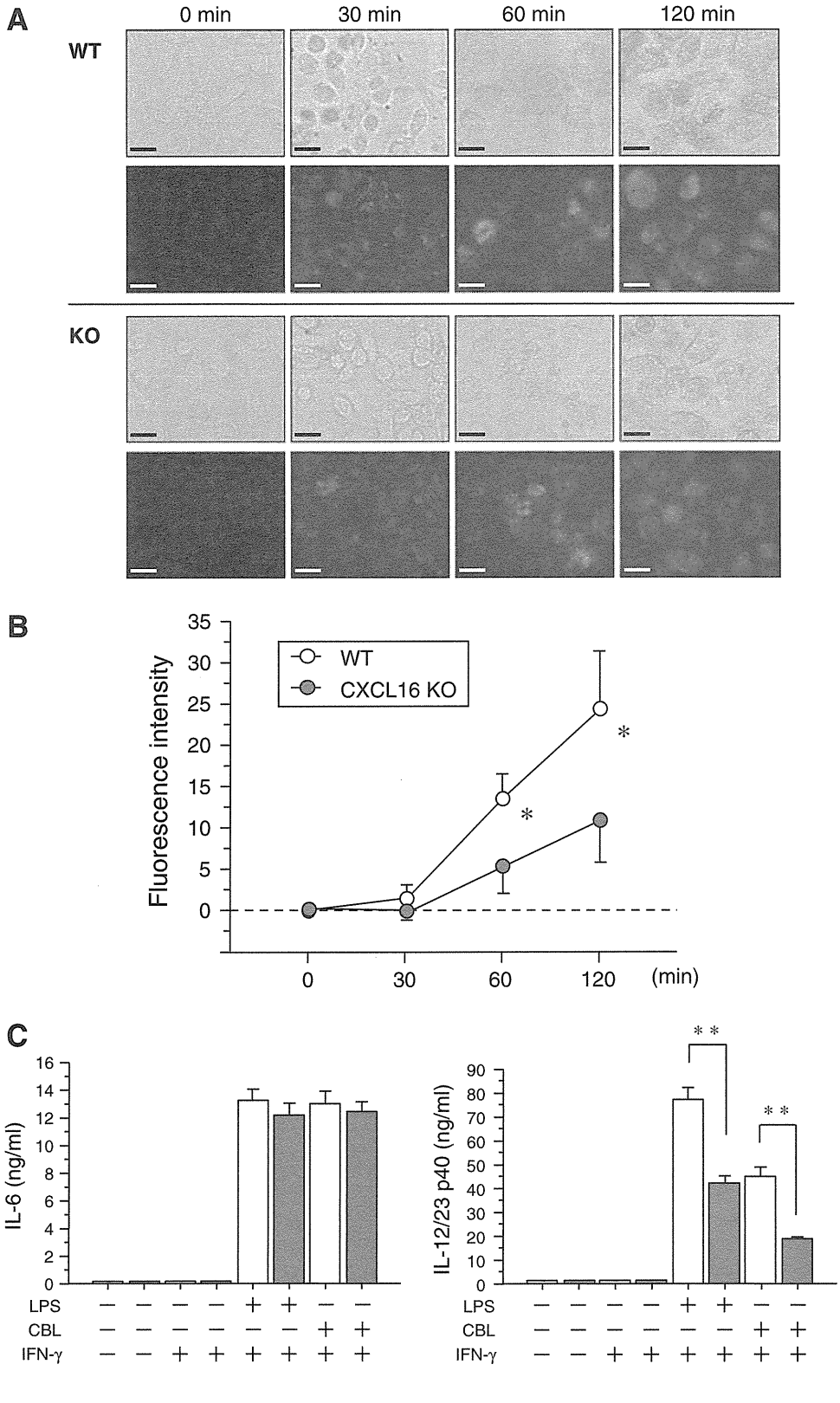

Figure 2 SR-PSOX/CXCL16 plays a role in phagocytosis of bacterial components and the production of interleukin 12 (IL-12) by macrophages. Thioglycollate-elicited peritoneal macrophages from SR-PSOX/CXCL16 knockout (KO) and wild-type (WT) mice were subjected to an ex vivo phagocytosis assay against bacteria. (A) Microscopic observation of phagocytosis was performed in peritoneal macrophages from WT mice (upper column) and SR-PSOX/CXCL16 KO mice (lower column). The upper and lower panels of each column show light microscopic findings and fluorescent images, respectively. Scale bars, 25 μm. (B) Fluorescence intensity, from which the fluorescence values of the no-cell background control wells were subtracted, was measured using a microplate reader at the indicated times. The statistical comparison of fluorescence intensity between WT (open circles) and SR-PSOX/CXCL16 KO macrophages (filled circles) was assessed by repeated measure analysis of variance followed by unpaired Student t test. (C) Peritoneal macrophages from WT (open bars) and SR-PSOX/CXCL16 KO mice (filled bars) were incubated with 500 U/ml interferon γ (IFNγ) for 16 h, followed by stimulation with 100 ng/ml lipopolysaccharide (LPS) or 30 μg caecal bacterial lysate (CBL) for 24 h, and the culture supernatants were analysed by ELISA to measure the concentrations of IL-6 and IL-12/23 p40. The results are expressed as means±SEM of the data from three independent experiments. The statistical difference was determined by unpaired Student t test. *$p<0.05$ and **$p<0.01$ between SR-PSOX/CXCL16 KO and WT macrophages.

streptavidin–HRP (1:1000; PerkinElmer, Waltham, Massachusetts, USA), treated with a tyramide signal amplification (TSA) biotin system (PerkinElmer), visualised with 3, 3′-diaminobenzidine tetrahydrochloride and counterstained with haematoxylin solution (Wako Pure Chemical, Osaka, Japan).

For immunofluorescence co-staining, cryosections (6 μm) of colonic tissue were fixed in cold acetone for 2 min and blocked with the Biotin Block system (DakoCytomation, Carpinteria, California, USA), Protein Block Serum-Free (DakoCytomation) and anti-CD16/32 mAb (1:500; eBioscience) to block endogenous biotin binding, non-specific protein binding and non-specific Fc binding, respectively. The sections were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD11b mAb (1:200; BD Pharmingen) and biotinylated anti-CXCL16 antibody (1:1000; R&D Systems) or goat IgG isotype control overnight at 4°C. After quenching, the sections were incubated with streptavidin–HRP (1:1000). The signals were enhanced using the TSA biotin system (PerkinElmer) according to the manufacturer's protocol and finally visualised by Alexa 594-conjugated streptavidin (1:2000; Invitrogen).

Furthermore, to evaluate the difference in cells recruited to colonic tissues of SR-PSOX/CXCL16 KO and WT mice with or without DSS-induced colitis, colonic tissues were incubated with FITC-conjugated CD3 (1:200), CD11b (1:100) and CD11c (1:100) (eBioscience), followed by nuclear counterstaining with 4`,6-diamidino-2-phenylindole (DAPI; 1:10000). Mean numbers of cells recruited to colonic tissues from five different microscopic fields under high power (×400) were calculated in each mouse, and means ± SEM from six mice of each group are shown. Images were recorded using a fluorescence microscope (Olympus).

Fluorescence in situ hybridisation (FISH)

The universal eubacterial oligonucleotide probe EUB-338 (5′-GCT GCC TCC CGT AGG AGT-3′) was synthesised and the 5′ end was labelled with carbocyanite dye (Cy5). The sections (4 μm thick) were deparaffinised and incubated with 50 ng of oligonucleotide probe in 10 μl of hybridisation buffer (containing 20% formamide, 0.9 M NaCl, 20 mM Tris–HCl (pH 7.2), 0.01% SDS) in a humid chamber overnight at 46°C. After washing with the same buffer, nuclear counterstaining was performed with DAPI (0.04 μg/ml). Slides were visualised by fluorescence microscopy with a Leica CW4000 system (Leica, Wetzlar, Germany).

Statistical analysis

All numerical data are expressed as means±SEM. The differences between groups were analysed by unpaired Student t test, Mann–Whitney U test and analysis of variance (ANOVA) for repeated measures. Parametric and non-parametric correlation was examined by the Pearson correlation coefficient test and the Spearman correlation test, respectively. The cumulative survival rate was calculated by the Kaplan–Meier method, and survival curves were compared by log-rank test. A p value <0.05 was considered significant.

RESULTS

Serum levels of SR-PSOX/CXCL16 increase in patients with active IBD

The serum levels of SR-PSOX/CXCL16 were significantly higher in patients with active CD and UC than in control subjects. The serum levels of SR-PSOX/CXCL16 were also significantly higher in patients with active CD and UC than in those with inactive CD and UC, respectively (figure 1A). Also, the serum levels of SR-PSOX/CXCL16 significantly correlated with clinical activities of both CD and UC (CADI and CAI; figure 1B,C). Considering the relationship among SR-PSOX/CXCL16, C-reactive protein (CRP) and clinical activities in CD and UC, SR-PSOX/CXCL16 might be a more suitable marker reflecting the disease activity of CD compared with that of UC.

SR-PSOX/CXCL16 plays a role in phagocytosis of bacterial components

SR-PSOX/CXCL16 is reported to be a chemokine expressed specifically on APCs such as macrophages and dendritic cells.[11–13] In particular, we focused on macrophages that play a critical role in the uptake of luminal antigens and examined the ability of macrophages from SR-PSOX/CXCL16 KO and WT mice to phagocytose bacteria ex vivo. Fluorescence microscopy showed that the uptake of *E coli* by macrophages from both SR-PSOX/CXCL16 KO mice and WT mice increased in a time-dependent manner (figure 2A). Measurement of fluorescence intensity revealed that the fluorescence value was significantly lower from 60 to 120 min in SR-PSOX/CXCL16 KO macrophages than in WT macrophages (figure 2B).

SR-PSOX/CXCL16 is involved in the production of IL-12 by macrophages

To compare cytokine production by macrophages between SR-PSOX/CXCL16 KO and WT mice, we measured the levels of IL-6 and IL-12/23 p40 in the supernatant from macrophages of mice stimulated with LPS or CBL after pretreatment with IFNγ. There was no difference in the production of IL-6 by macrophages between SR-PSOX/CXCL16 KO and WT mice. In contrast, the production of IL-12/23 p40 by macrophages was

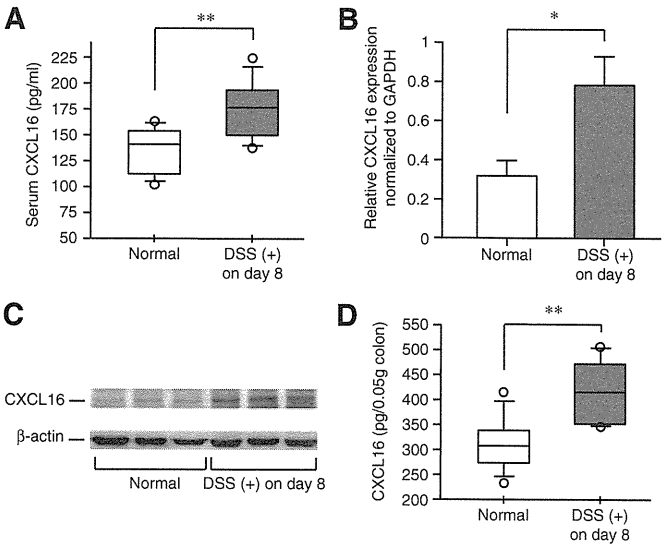

Figure 3 SR-PSOX/CXCL16 levels are higher in mice with dextran sulfate sodium (DSS)-induced colitis. (A) The serum levels of SR-PSOX/CXCL16 in mice with DSS-induced colitis on day 8 and control mice were measured by ELISA. (B). The gene expression of SR-PSOX/CXCL16 in colonic tissues with or without DSS-induced colitis was determined by quantitative real-time reverse transcription–PCR (RT–PCR) and was normalised to glyceraldehyde phosphate dehydrogenase (GAPDH). (C). The production of SR-PSOX/CXCL16 in colonic tissues with or without DSS-induced colitis was investigated by western blot analysis. (D) SR-PSOX/CXCL16 concentrations in supernatants of colon fragment cultures were measured by ELISA. The results are expressed as means±SEM (n=10 in each group). (A), (B) and (D) The statistical difference was determined by unpaired Student t test. *p<0.05 and **p<0.01 between mice with DSS-induced colitis and normal controls.

significantly lower in SR-PSOX/CXCL16 KO mice than in WT mice (figure 2C). We also observed a significant difference in IL-12/23 p40 production by macrophages between SR-PSOX/CXCL16 KO and WT mice even without pretreatment with IFNγ (data not shown).

Expression of SR-PSOX/CXCL16 increases in mice with DSS-induced colitis

Next, we investigated the in vivo expression of SR-PSOX/CXCL16 in the DSS-induced colitis model. The serum levels of SR-PSOX/CXCL16 were significantly higher in mice with DSS-induced colitis than in normal controls (figure 3A). To examine whether the expression of SR-PSOX/CXCL16 increases in inflamed tissues, we analysed the colonic tissues of mice with or without DSS-induced colitis. The gene expression of SR-PSOX/CXCL16 was significantly higher in colonic tissues of DSS-induced colitis than in normal colon (figure 3B). Western blot analysis and ELISA also revealed that SR-PSOX/CXCL16 expression was significantly higher in colonic tissues of DSS-induced colitis than in normal colon (figure 3C,D).

To identify the cells that mainly express SR-PSOX/CXCL16 in colonic tissues, we performed immunohistochemical analysis and immunofluorescent co-staining. The follicular-associated epithelia of Peyer's patches, used as a control, were positive for SP-PSOX/CXCL16, as reported previously (figure 4A, right upper panel).[32] SR-PSOX/CXCL16-expressing cells were increased markedly in colonic tissues of mice with DSS-induced colitis compared with normal colons, and these cells were observed from the mucosae to the submucosa (figure 4A, right lower panel). Immunofluorescent images revealed that SR-PSOX/CXCL16-expressing cells were mainly CD11b-positive cells (figure 4B).

Activity of DSS-induced colitis is reduced in SR-PSOX/CXCL16 KO mice

To investigate the role of SR-PSOX/CXCL16 in colonic inflammation, we compared SR-PSOX/CXCL16 KO and WT mice with DSS-induced colitis. Before the analysis, we confirmed that there was no difference in the subsets of lymphocytes between SR-PSOX/CXCL16 KO mice and WT mice in the static state (Supplementary figure 1 online). The amount of body weight

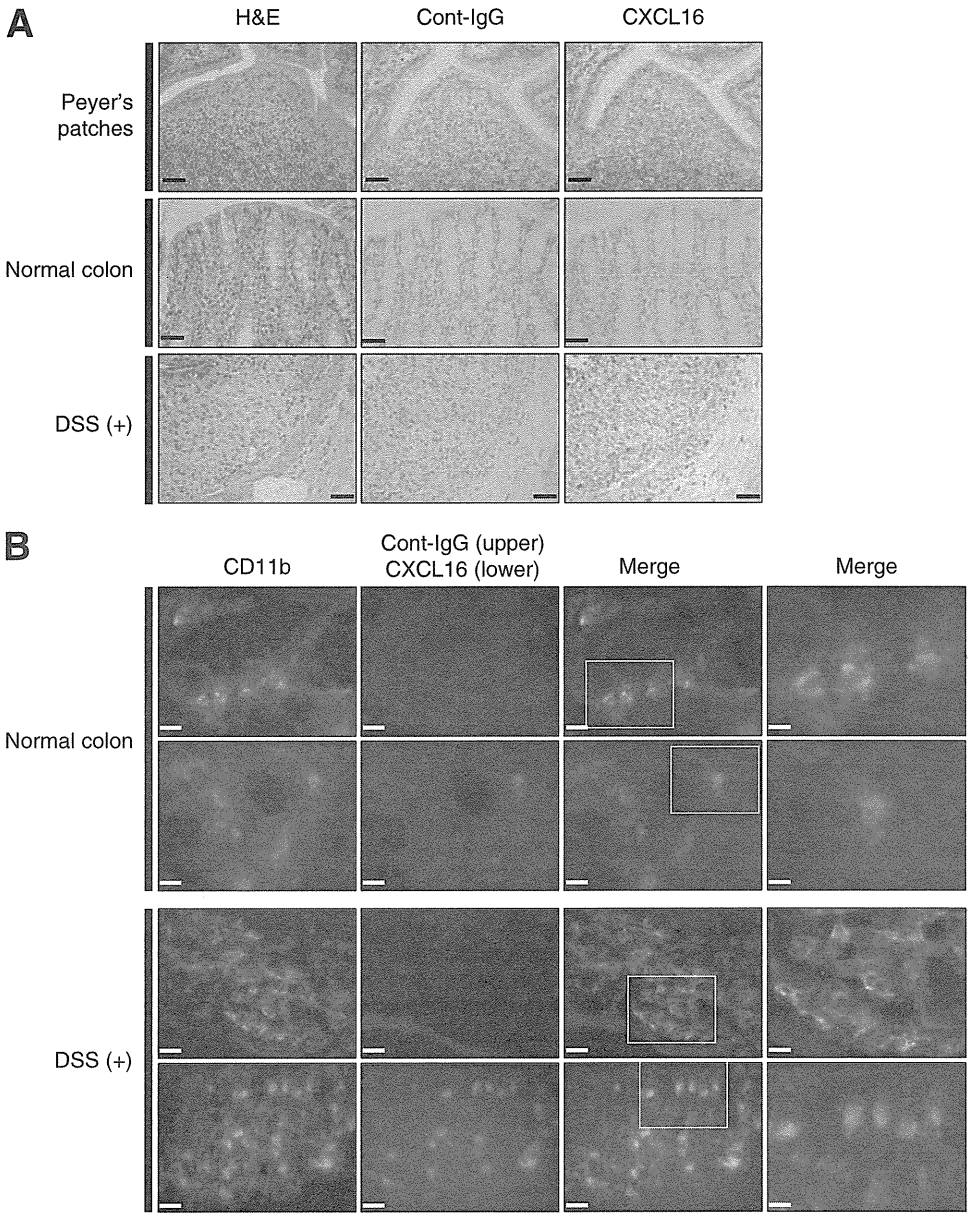

Figure 4 SR-PSOX/CXCL16 is expressed predominantly on macrophages in colonic tissues of mice with dextran sulfate sodium (DSS)-induced colitis. (A) Immunostaining was performed in Peyer's patches as a positive control, normal colons and colons with 3% DSS-induced colitis. Serial sections of each tissue were stained with H&E, control goat immunoglobulin G (IgG) and anti-mouse SR-PSOX/CXCL16 monoclonal antibody. (B). Immunofluorescent staining was performed in normal colons and colons with 3% DSS-induced colitis using antibodies against CD11b (green), SR-PSOX/CXCL16 (red) and control goat IgG. The merged images and their magnified images are shown. Scale bars, 50 μm (A), 20 μm (B, left 3 lanes) and 10 μm (B, right lane).

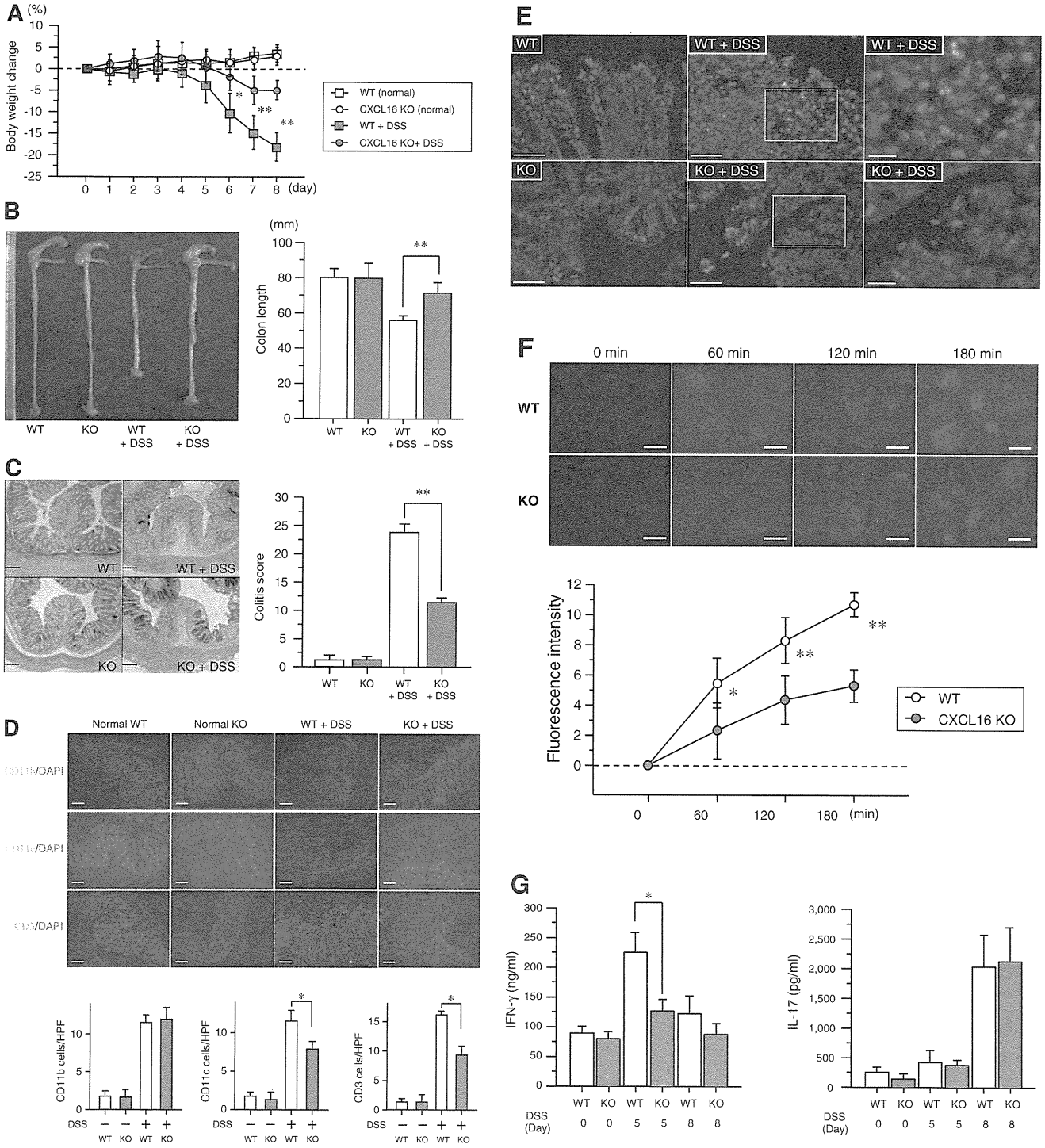

Figure 5 Activity of dextran sulfate sodium (DSS)-induced colitis is lower in SR-PSOX/CXCL16 knockout (KO) mice. (A) Serial change in body weight in SR-PSOX/CXCL16 KO and wild-type (WT) mice with or without 3% DSS-induced colitis. Data are expressed as the percentage change from the starting body weight. (B) Representative image and colonic length in SR-PSOX/CXCL16 KO and WT mice with or without 3% DSS-induced colitis on day 8. (C) Representative histological findings and the scores of colonic inflammation of SR-PSOX/CXCL16 KO and WT mice with or without 3% DSS-induced colitis on day 8. Scale bars, 100 μm. (D) Colonic tissues of SR-PSOX/CXCL16 KO and WT mice with or without 3% DSS-induced colitis were incubated with fluorescein isothiocyanate (FITC)-conjugated CD3, CD11b and CD11c, followed by nuclear counterstaining with 4′,6-diamidino-2-phenylindole (DAPI). Scale bars, 100 μm. (E) Fluorescent in situ hybridization analysis was performed with colonic tissues of SR-PSOX/CXCL16 KO and WT mice with or without 3% DSS-induced colitis using eubacterial oligonucleotide probe EUB-338 (red), followed by DAPI (blue). Scale bars, 50 μm (left two lanes) and 20 μm (right lane). (F) Colonic macrophages from SR-PSOX/CXCL16 KO and WT mice were subjected to an ex vivo phagocytosis assay against bacteria. Scale bars, 25 μm. (G) MLN cells from SR-PSOX/CXCL16 KO and WT mice on days 0, 5 and 8 after administration of 3% DSS were cultured with immobilised anti-CD3 plus CD28. Supernatants were collected after 72 h and subjected to ELISA to measure the concentration of interferon γ (IFNγ) and interleukin 17 (IL-17). (A)–(D), (F) and (G) The results are expressed as means±SEM (n=10–12 in each group). The statistical comparison was assessed by repeated measure analysis of variance followed by unpaired Student t test (A) and (F). The statistical difference was determined by unpaired Student t test (B), (D) and (G) or Mann–Whitney U test (C). *p<0.05 and **p<0.01 between SR-PSOX/CXCL16 KO mice and WT mice with DSS-induced colitis.

loss was significantly less in SR-PSOX/CXCL16 KO mice than in WT mice from 6 to 8 days after DSS administration (figure 5A). The colon was significantly longer in SR-PSOX/CXCL16 KO mice with DSS-induced colitis than in WT mice with DSS-induced colitis (figure 5B). The histological findings on day 8 after DSS administration in WT mice revealed severe epithelial destruction, remarkable infiltration of inflammatory cells with submucosal oedema, and crypt loss (figure 5C, right upper panel). In contrast, these findings were mild in SR-PSOX/CXCL16 KO mice (figure 5C, right lower panel). The total colitis score was significantly lower in SR-PSOX/CXCL16 KO mice with DSS-induced colitis than in WT mice with DSS-induced colitis (figure 5C). Furthermore, fluorescent immunohistochemistry showed that the numbers of CD11c- and CD3-positive cells were significantly lower in colonic tissues of SR-PSOX/CXCL16 KO mice with DSS-induced colitis than in WT mice with DSS-induced colitis, despite no significant difference of the number of CD11b-positive cells between these two groups (figure 5D).

SR-PSOX/CXCL16 is involved in bacterial invasion and phagocytosis of bacterial components in inflamed colonic tissues

FISH with a universal oligonucleotide probe was performed to elucidate the difference in bacterial invasion of colonic tissues between SR-PSOX/CXCL16 KO and WT mice with/without colitis. FISH analysis showed that fewer bacteria invaded colonic tissues in SR-PSOX/CXCL16 KO mice with colitis compared with WT mice with colitis (figure 5E). Additionally, we investigated the phagocytic activity of colonic macrophages. Similar to the data with peritoneal macrophages, the fluorescence value was significantly lower from 60 to 180 min in SR-PSOX/CXCL16 KO colonic macrophages than in WT colonic macrophages (figure 5F).

SR-PSOX/CXCL16 is related to the Th1 but not Th17 immune response in DSS-induced colitis

Next, we measured cytokine production by MLN cells from both SR-PSOX/CXCL16 KO and WT mice with DSS-induced colitis. The production of IFNγ on day 5 after DSS administration was significantly lower in SR-PSOX/CXCL16 KO mice than in WT mice (figure 5G). In contrast, the production of IL-17 did not differ significantly between SR-PSOX/CXCL16 KO and WT mice with DSS-induced colitis throughout the experiment (figure 5G).

Administration of SR-PSOX/CXCL16 mAb attenuates experimental murine colitis

To assess the neutralising effect of a mAb to SR-PSOX/CXCL16 in mice with colonic inflammation, we analysed two experimental murine colitis models: DSS-induced colitis as an epithelial injury model and TNBS-induced colitis as a Th1-mediated colitis model.[33] The body weight of mice with DSS-colitis treated with control IgG decreased and reached the lowest level on day 9, and gradually increased thereafter, although complete recovery was not obtained even on day 14. In contrast, in mice treated with SR-PSOX/CXCL16 mAb the body weight

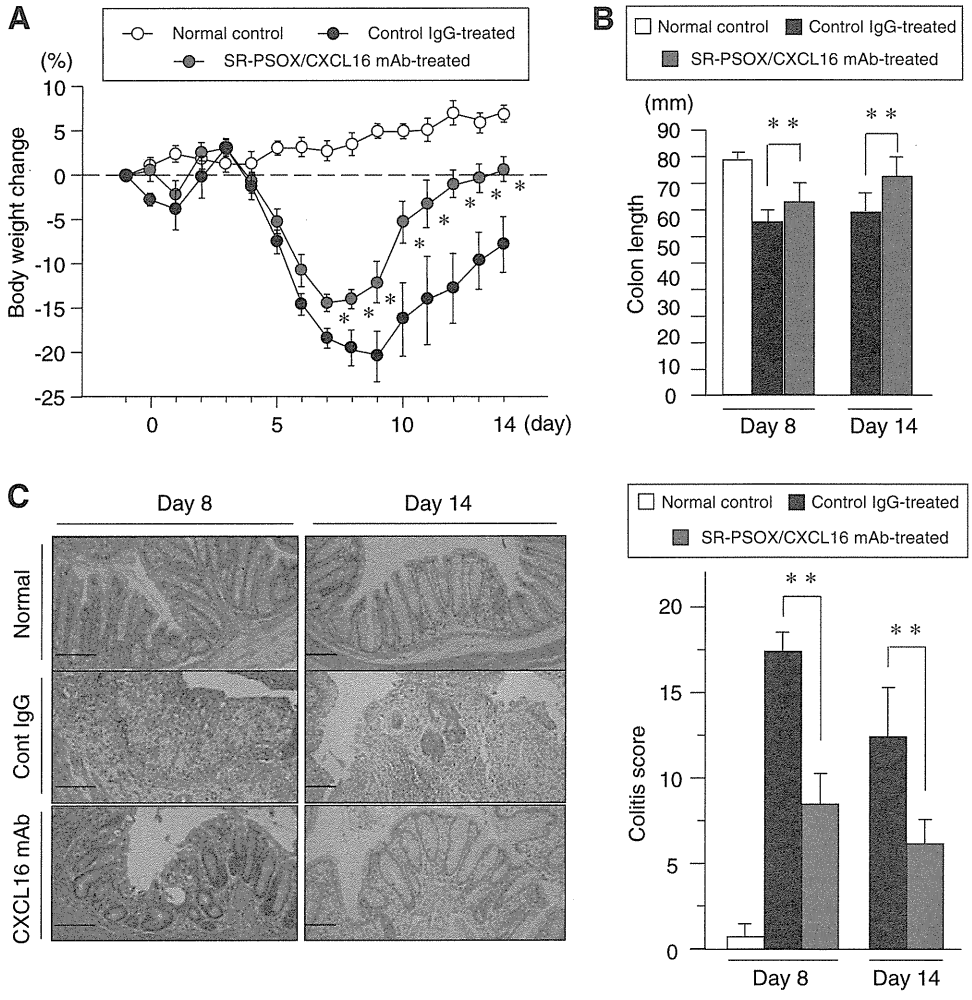

Figure 6 Administration of SR-PSOX/CXCL16 monoclonal antibody (mAb) attenuates dextran sulfate sodium (DSS)-induced colitis. A 500 μg aliquot of SR-PSOX/CXCL16 mAb or an equal amount of control rat immunoglobulin (IgG) was administered to C57BL/6 mice with 3% DSS-induced colitis by intraperitoneal injection once a day from day 1 to day 7. (A) Serial change in body weight in normal control mice (open circles), control IgG-treated mice with DSS-induced colitis (filled circles) and SR-PSOX/CXCL16 mAb-treated mice with DSS-induced colitis (grey circles). The data are expressed as the percentage change from the starting body weight. (B) Colonic length on day 8 and day 14 after administration of DSS. (C) Representative histological findings and the scores of colonic inflammation on day 8 and day 14. Scale bars, 100 μm. The results are expressed as means±SEM (n=12 in each group). (A) The statistical comparison was assessed by repeated measure analysis of variance followed by unpaired Student t test. (B) and (C) The differences of colon length and colitis score between the groups were determined by unpaired Student t test and Mann–Whitney U test, respectively. *$p<0.05$ and **$p<0.01$ between SR-PSOX/CXCL16 mAb-treated and control IgG-treated mice with DSS-induced colitis.

decreased to the lowest level on day 7 and recovered to a level similar to that before DSS administration on day 14. The body weight of mice treated with SR-PSOX/CXCL16 mAb was significantly higher from day 7 to 14 than that of IgG-treated control mice (figure 6A). Furthermore, administration of SR-PSOX/CXCL16 mAb significantly attenuated the shortening of colonic length (figure 6B), and significantly reduced colonic damage and the colitis score of mice with DSS-induced colitis on both day 8 and day 14 (figure 6C).

In addition, in TNBS-induced colitis, administration of SR-PSOX/CXCL16 mAb significantly ameliorated the body weight change on day 4 (figure 7A). Histological findings showed severe epithelial destruction, marked infiltration of inflammatory cells with mucosal oedema and remarkable loss of cryptal cells in control IgG-treated mice. In contrast, these colonic inflammatory findings were attenuated in SR-PSOX/CXCL16 mAb-treated mice. The colitis score was significantly lower in SR-PSOX/CXCL16 mAb-treated mice than in control IgG-treated mice (figure 7B). Furthermore, the overall survival rate of mice with TNBS-induced colitis treated with SR-PSOX/CXCL16 mAb was significantly higher than that of control IgG-treated mice (78.7% vs 38.9%; p=0.04; figure 7C).

DISCUSSION

Recent genetic approaches for elucidating the pathogenesis of IBD revealed that abnormality of the genes related to the innate immune response by recognising and/or processing bacterial components is involved in the development of IBD.[34–37] Kamada *et al* suggested that the abnormal response of intestinal macrophages to commensal bacteria results in chronic intestinal inflammation.[4] Therefore, the control of the abnormal innate immune response of APCs to commensal bacteria is important in the treatment of IBD. Indeed, we showed that macrophage-targeting treatment ameliorates colonic inflammation in an experimental colitis model.[5] Taken together, targeting molecules related to macrophages appears to be a promising approach for the treatment of IBD. SR-PSOX/CXCL16 may be one such candidate molecule, because it is mainly expressed in APCs.

First, we found that the serum level of SR-PSOX/CXCL16 was significantly higher in patients with active IBD as reported previously,[38] and moreover that the level correlated with the disease activity in patients with IBD. Analysis of correlation between disease activity and SR-PSOX/CXCL16 or CRP suggests that the serum SR-PSOX/CXCL16 might be a suitable biomarker for evaluating disease activity of CD rather than UC.

Furthermore, we investigated the serum concentration and tissue expression of SR-PSOX/CXCL16 in mice with DSS-induced colitis. Similar to human IBD, the serum level of SR-PSOX/CXCL16 and its expression in colonic tissue was significantly higher in mice with DSS-induced colitis than in normal mice. In addition, SR-PSOX/CXCL16 was expressed mainly on CD11b-positive cells and markedly increased in the colonic mucosa of mice with DSS-induced colitis, although these cells were barely observed in the colonic mucosa under normal conditions. Previous reports have shown that several inflammatory cytokines including IFNγ, tumour necrosis factor α (TNFα) and IL-18 induce the expression of SR-PSOX/CXCL16.[39–41] Thus, increased concentration of various inflammatory cytokines in the inflamed colonic mucosa may contribute to enhanced expression of SR-PSOX/CXCL16 on macrophages.

Next, to evaluate the role of SR-PSOX/CXCL16 in both macrophage phagocytic activity and cytokine production, we examined phagocytosis and bacteria stimulated-cytokine production in peritoneal macrophages from SR-PSOX/CXCL16 KO mice in vitro. Our data clearly demonstrated that both the phagocytic ability and IL-12 production of macrophages of

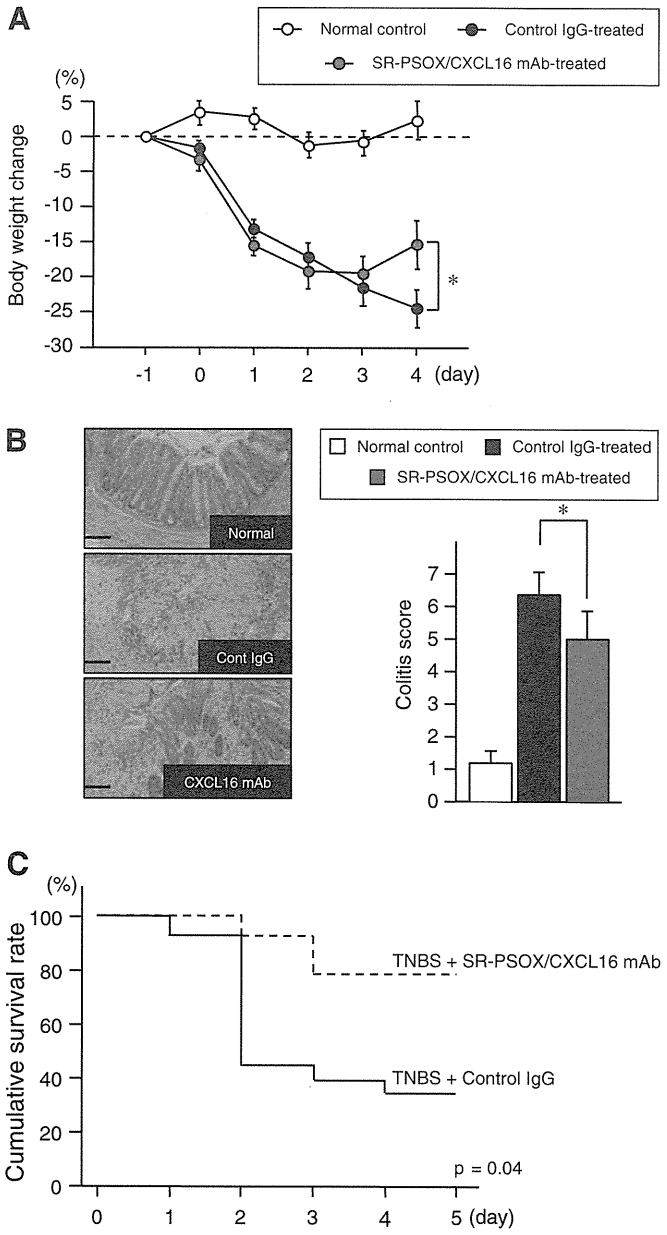

Figure 7 Administration of SR-PSOX/CXCL16 monoclonal antibody (mAb) attenuates trinitrobenzene sulfonic acid (TNBS)-induced colitis. A 500 μg aliquot of SR-PSOX/CXCL16 mAb or an equal amount of control rat immunoglobulin G (IgG) was given to SJL/J mice with TNBS-induced colitis by intraperitoneal injection once a day from day 1 to day 3. (A) Serial change in body weight in normal control mice (open circles), control IgG-treated mice with TNBS-induced colitis (filled circles) and SR-PSOX/CXCL16 mAb-treated mice with TNBS-induced colitis (grey circles). The data are expressed as the percentage change from the starting body weight. (B) Representative histological findings and the scores of colonic inflammation on day 4. Scale bars, 100 μm. The results are expressed as means±SEM (n=8 in each group). The difference of body weight change and colitis score between groups was assessed by repeated measure analysis of variance followed by unpaired Student t test and Mann–Whitney U test, respectively. *p<0.05 between SR-PSOX/CXCL16 mAb-treated and control IgG-treated mice with TNBS-induced colitis. (C) Cumulative survival rate of mice with TNBS-colitis treated with SR-PSOX/CXCL16 or control IgG was calculated by the Kaplan–Meier method, and survival curves were compared by log-rank test.

SR-PSOX/CXCL16 KO mice was significantly impaired. Of note, LPS- and CBL-induced IL-12 production was significantly reduced in SR-PSOX/CXCL16 KO mice in the presence of IFNγ. This appears reasonable, because IFNγ has been reported to enhance SR-PSOX/CXCL16 expression.[39]

The present study clearly showed that SR-PSOX/CXCL16 KO mice had reduced activity of DSS-induced colitis. Moreover, SR-PSOX/CXCL16 mAb ameliorated colitis in two different experimental models. These data indicate that SR-PSOX/CXCL16 plays important roles in the development of colitis. The DSS-induced colitis model is characterised by direct epithelial injury and subsequent activation of macrophages,[33] and SR-PSOX/CXCL16 is reported to be involved in DSS-induced IL-1β production by macrophages.[42] Therefore, the DSS-induced colitis model is suitable for investigating the interaction between luminal bacteria and macrophages expressing SR-PSOX/CXCL16. In our current study, SR-PSOX/CXCL16 was expressed predominantly on CD11b-positive cells at subepithelial sites of the inflamed colonic mucosa. In addition, we found that bacterial invasion of the lamina propria in the colitic mucosa was reduced in SR-PSOX/CXCL16 KO mice. SR-PSOX/CXCL16 has been reported to act as a scavenger receptor that mediates adhesion and phagocytosis of bacteria by APCs.[14] In this connection, we demonstrated here that both peritoneal and intestinal macrophages of SR-PSOX/CXCL16 KO mice had reduced ability to phagocytose bacterial antigens. Taken together, our data suggest that SR-PSOX/CXCL16 exerts its colitogenic action at least in part by promoting bacterial uptake into macrophages in the colonic mucosa.

In this study, SR-PSOX/CXCL16 mAb also ameliorated TNBS-induced colitis, a Th1-mediated colitis model. An interesting finding in this study is that SR-PSOX/CXCL16 KO macrophages had an impaired ability to produce IL-12 in response to not only LPS but also commensal bacterial antigens, although their production of IL-6 was unaffected. Moreover, we observed that MLN cells from SR-PSOX/CXCL16 KO mice with DSS-induced colitis showed reduced production of IFNγ but not of IL-17. Because IL-6 is essential for the induction of Th17 cells, the lack of difference in IL-6 production by macrophages or IL-17 production by MLN cells between SR-PSOX/CXCL16 KO and WT mice suggests that SR-PSOX/CXCL16 is not involved in the Th17-mediated immune response. Taken together, our data indicate that SR-PSOX/CXCL16 plays a colitogenic role by enhancing the Th1 immune response. Of note, IL-17A had a protective role in the $CD45RB^{hi}$ transfer model of colitis and suppressed the induction of T-bet in maturing Th1 cells.[43] Thus, targeting SR-PSOX/CXCL16 seems to be an ideal treatment for preventing Th1-mediated colitis, without affecting the IL-17-mediated immune response.

In conclusion, our present data clearly demonstrated that SR-PSOX/CXCL16 plays a critical role in the development of colonic inflammation probably by both activating uptake of commensal bacteria and enhancing the Th1 immune response. SR-PSOX/CXCL16 may be a therapeutic target for patients with IBD.

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Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of the Kyoto University Hospital Ethics Committee.

Provenance and peer review Not commissioned; externally peer reviewed.

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Editor's quiz: GI snapshot

Left lower quadrant abdominal pain caused by an IUCD

CLINICAL PRESENTATION

A previously healthy 42-year-old woman presented with mild left lower quadrant abdominal pain. There was no relevant medical or family history except she had a T-shaped copper intrauterine contraceptive device (IUCD) inserted 10 years previously. The remainder of her obstetric history was non-specific and included one normal vaginal delivery. Physical examination and laboratory findings at the time of presentation were unremarkable.

Plain radiographs of the abdomen identified the IUCD in the pelvic cavity (figure 1); however, the IUCD was not detected during gynaecological ultrasonography. Further abdominal CT scanning did not reveal any definite focal lesions and the IUCD was not identified (figure 2).

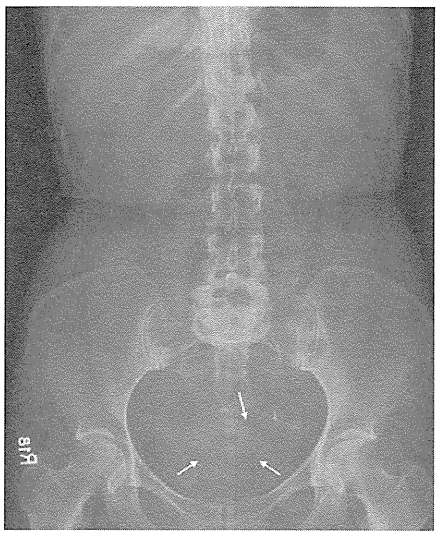

Figure 1 Plain abdominal radiograph showed the T-shaped intrauterine contraceptive device (IUCD) in the left pelvic cavity. The surrounding fat planes of the uterus were identified (arrows) and the device appeared separate from the fat planes.

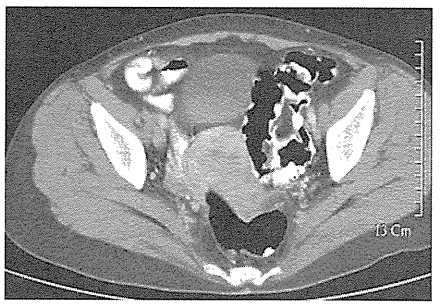

Figure 2 Contrast-enhanced CT scan using the soft tissue window setting did not identify any definite focal lesions.

QUESTION

What is your diagnosis?

What additional tests are indicated and how should the patient be managed?

See page 1562 for the answer

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SR-PSOX/CXCL16 plays a critical role in the progression of colonic inflammation

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COX-2 inhibition alters the phenotype of tumor-associated macrophages from M2 to M1 in *Apc*$^{Min/+}$ mouse polyps

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Macrophages are a major component of tumor stroma. Tumor-associated macrophages (TAMs) show anti- (M1) or protumor (M2) functions depending on the cytokine milieu of the tumor microenvironment. Cyclooxygenase-2 (COX-2) is constitutively expressed in a variety of tumors including colorectal cancer. TAMs are known to be a major source of COX-2 in human and mice intestinal tumors. COX-2 inhibitor reduces the number and size of intestinal adenomas in familial adenomatous polyposis patients and *Apc*$^{Min/+}$ mice. Although COX-2 inhibitor is thought to regulate cancer-related inflammation, its effect on TAM phenotype remains unknown. Here, we examined the effects of COX-2 inhibition on TAM phenotype and cytokine expression both *in vivo* and *in vitro*. Firstly, the selective COX-2 inhibitor celecoxib changed the TAM phenotype from M2 to M1, in proportion to the reduction in number of *Apc*$^{Min/+}$ mouse polyps. Concomitantly, the expression of M1-related cytokine interfron (IFN)-γ was significantly upregulated by celecoxib, although the M2-related cytokines interleukin (IL)-4, IL-13 and IL-10 were not significantly altered. Secondly, IFN-γ treatment attenuated M2 phenotype of mouse peritoneal macrophages and oriented them to M1 even in the presence of M2-polarizing cytokines such as IL-4, IL-13 and IL-10. Thus, our results suggest that COX-2 inhibition alters TAM phenotype in an IFN-γ-dependent manner and subsequently may reduce intestinal tumor progression.

Introduction

Macrophages are a major component of the leukocyte infiltrates in various tumor stroma and macrophages that infiltrate tumors are called tumor-associated macrophages (TAMs) (1,2). TAMs have been recognized as a part of the inflammatory circuits that promote tumor progression (1,2). Since macrophages have functional plasticity and can change their activation status in response to the microenvironment (3,4), the concept of phenotypic heterogeneity of macrophages has been strengthened with the classification of M1 (classically activated) and M2 (alternatively activated) phenotypes in analogy with the Th1 and Th2 dichotomy (3,5,6). It is considered that when macrophages are exposed to lipopolysaccharides and interfron (IFN)-γ, they are polarized to the M1 phenotype and have antitumor functionality. When they are exposed to Th2 cytokines, such as interleukins (IL)-4, IL-13 and IL-10, they are polarized to the M2 phenotype and support cell proliferation and tumor growth (5,7). TAM is considered as a polarized M2 macrophage population in most mouse and human tumors (e.g. breast, prostate, glioma and lymphoma) (5,8).

Several studies have demonstrated that cyclooxygenase-2 (COX-2) is constitutively expressed in colorectal cancer, predominantly by stromal cells (9–18). Macrophages are one of the major sources of COX-2 in colorectal cancer (11,15–19). COX-2 promotes the progression of colorectal cancer through prostaglandin production and inhibition of COX-2 by traditional nonsteroidal anti-inflammatory drugs or selective COX-2 inhibitors reduces the number and size of adenomas in familial adenomatous polyposis patients and in *Apc* knockout mice (9,12–14,20–25). Although previous studies have shown that COX-2 inhibitors affect cytokine expression profiles and tumor microenvironments (26), the effect of COX-2 inhibition on TAM phenotype remains unclear.

To examine the effect of COX-2 inhibition on TAMs, we investigated the status of TAMs in *Apc*$^{Min/+}$ mouse intestinal polyps and concomitant cytokine expression profiles with or without selective COX-2 inhibitors. In addition, we studied the effect of COX-2 inhibition and various cytokines on mouse peritoneal macrophages in terms of the M1/M2 phenotype.

Abbreviations: COX-2, cyclooxygenase-2; IFN, interfron; IL, interleukin; iNOS, inducible nitric oxide synthase; MR, mannose receptor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TAM, tumor-associated macrophage.

†These authors contributed equally to this work.

Materials and methods

Mouse models

Apc$^{Min/+}$ mice were obtained from Jackson Laboratory (Bar Harbor, ME), and 9- to 10-week-old male *Apc*$^{Min/+}$ mice were treated with either drug-free chow or celecoxib-mixed chow (Pfizer, Groton, CT) for 8 weeks. Celecoxib were prepared in chow with a dose of 330, 66 or 6.6 μg/body wt g/day. After the mice were killed, their intestinal polyps were counted as described previously (27). All experiments were approved by the Animal Research Committee of Kyoto University and performed in accordance with the Japanese government regulations.

Histological analysis and immunofluorescence/immunohistochemistry stainings

For histological analysis, intestinal samples were fixed overnight in 4% paraformaldehyde, embedded in paraffin and sectioned at 5 μm thickness. Subsequently, sections were deparaffinized, rehydrated and stained with hematoxylin and eosin. For immunohistochemistry, sections were incubated with primary antibody (rat anti-mouse F4/80, 1:100; Abcam, Cambridge, MA) overnight and with the biotinylated secondary antibody followed by incubation with the avidin–biotin–peroxidase complex (Vector Labs, Southfield, MI). The sections were labeled with peroxidase and colored with diaminobenzidine substrate (Dako, Glostrup, Denmark). For immunofluorescence, intestinal samples were embedded in OCT compound (Sakura, Tokyo, Japan) and frozen at −80°C. These frozen samples were sectioned at 6 μm, fixed in methanol, washed with phosphate-buffered saline (PBS) and then incubated with the primary antibodies [rat anti-mouse F4/80 (1:100; Abcam), rabbit anti-mouse F4/80 (1:100; Abcam), rat anti-mouse mannose receptor (MR) (1:25; Hycult biotech, Uden, Netherlands), rabbit anti-mouse inducible nitric oxide synthase (iNOS) (1:100; Abcam), rabbit anti-mouse COX2 (1:100; Abcam), Alexa Fluor 488 conjugated hamster anti-mouse CD3ε (1:100; eBioscience, San Diego, CA), mouse anti-mouse Ncam (1:100; Abcam) or rat anti-mouse IFN-γ (1:400; eBioscience)] overnight at 4°C and washed again with PBS. Washed sections were treated with fluorescence-conjugated secondary antibodies (Invitrogen, Carlsbad, CA) for 2 h.

Cell preparations

Mouse peritoneal macrophages were isolated from 8-week-old female C57BL/6J mice. Peritoneal cells were harvested by peritoneal lavage with 10 ml PBS. Cells were resuspended and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum, 100 mg/ml of penicillin, 100 mg/ml of streptomycin and 1.25 μg/ml of amphotericin B. 1×10^6 peritoneal cells were seeded into a 12-well dish and incubated for 2 h. Then, cells were washed in PBS and recultured in Dulbecco's modified Eagle's medium without fetal calf serum. To inhibit COX-2 activity, 10 μM of celecoxib was added to the culture medium with 20 ng/ml of mouse recombinant IL-4 (PeproTech, Rocky Hill, NJ) and 20 ng/ml of mouse recombinant IL-13 (PeproTech) or with 20 ng/ml of mouse recombinant IL-10 (R&D Systems, Minneapolis, MN). For the IFN-γ addition experiment, 20 ng/ml of mouse

recombinant IFN-γ (PeproTech) was added to the cells with 20 ng/ml of IL-4 and 20 ng/ml of IL-13 or with 20 ng/ml of IL-10. Cells were collected 4 h after stimulation.

RNA isolation and quantitative reverse transcription–polymerase chain reaction

We isolated RNA from $Apc^{Min/+}$ mice and mouse peritoneal macrophages using a TRIzol reagent (Invitrogen) according to the manufacturer's instructions and then synthesized single-stranded complementary DNA from 1 μg of total RNA using Superscript III (Invitrogen). We performed quantitative polymerase chain reaction (PCR) using the LightCycler FastStart DNA Master SYBR Green 1 kit (Roche Diagnostics, Osaka, Japan). The amplification conditions included 10 s of denaturation at 95°C, 5 s of annealing at 57°C and 10 s of extension at 72°C for a total of 45 cycles. Quantitative reverse transcription (qRT)–PCR primers were as follows: mouse GAPDH-forward, AGGTCGGTGTGAACGGATTTG, mouse GAPDH-reverse, TGTAGACCATGTAGTTGAGGTCA; mouse Arginase 1-forward, TGGCTTGCGAGACGTAGAC, mouse Arginase 1-reverse, GCTCAGGTGAATCGGCCTTTT; mouse MR-forward, GCTGAATCCCAGAAATTCCGC, mouse MR-reverse, ATCACAGGCATACAGGGTGAC; mouse iNOS-forward, GTTCTCAGCCCAACAATACAAGA, mouse iNOS-reverse, GTGGACGGGTCGATGTCAC; mouse Ym1-forward, TTATCCTGAGTGACCCTTCTAAG, mouse Ym1-reverse, TCATTACCCTGATAGGCATAGG; mouse Trem2-forward, CTGGAACCGTCACCATCACTC, mouse Trem2-reverse, CGAAACTCGATGACTCCTCGG; mouse CXCL10-forward, CCAAGTGCTGCCGTCATTTTC, mouse CXCL10-reverse, GGCTCGCAGGGATGATTTCAA; mouse IFN-γ-forward, ATGAACGCTACACACTGCATC, mouse IFN-γ-reverse, CCATCCTTTTGCCAGTTCCTC; mouse IL-4-forward, GGTCTCAACCCCCAGCTAGT, mouse IL-4-reverse, GCCGATGATCTCTCTCAAGTGAT; mouse IL-10-forward, GCTCTTACTGACTGGCATGAG, mouse IL-10-reverse, CGCAGCTCTAGGAGCATGTG; mouse IL-13-forward, GGATATTGCATGGCCTCTGTAAC, mouse IL-13-reverse, AACAGTTGCTTTGTGTAGCTGA; mouse IL-12-forward, ACTCTGCGCCAGAAACCTC, mouse IL-12-reverse, CACCCTGTTGATGGTCACGAC; mouse CD4-forward, AGGTGATGGGACCTACCTCTC, mouse CD4-reverse, GGGGCCACCACTTGAACTAC; mouse CD8a-forward, CCGTTGACCCGCTTTCTGT, mouse CD8a-reverse, CGGCGTCCATTTTCTTTGGAA; mouse Nkg2d-forward, ACTCAGAGATGAGCAAATGCC, mouse Nkg2d-reverse, CAGGTTGACTGGTAGTTAGTGC.

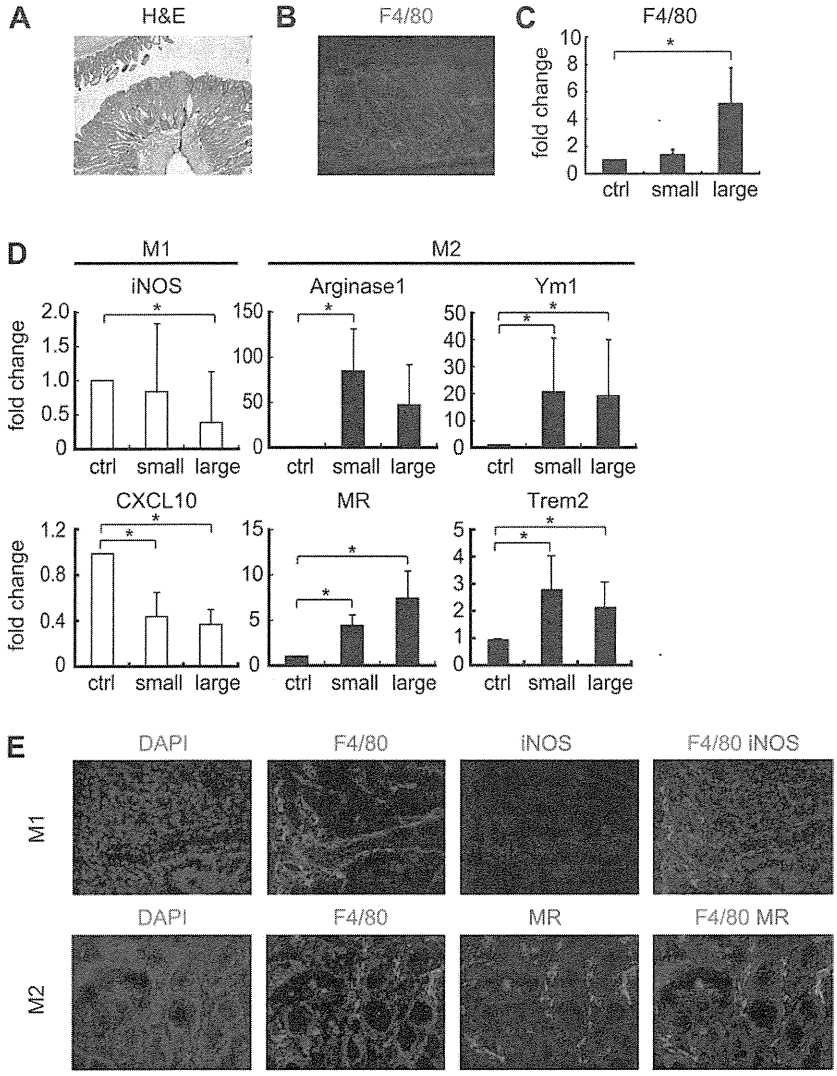

Fig. 1. TAMs infiltrating $Apc^{Min/+}$ mouse polyps were polarized to the M2 phenotype. (**A**) Representative hematoxylin and eosin staining of small intestinal polyp of $Apc^{Min/+}$ mouse. Original magnification, ×100. (**B**) $Apc^{Min/+}$ mouse polyp stroma was infiltrated with F4/80-positive TAMs (green). (**C**) qRT–PCR showed that expression of F4/80 was upregulated in large polyps. Data represent fold induction of mRNA expression compared with histologically normal mucosa (ctrl) ($n = 6$). $^*P < 0.05$. (**D**) The expression of M1 and M2 genes in small or large polyps was evaluated by qRT–PCR. In intestinal polyps, M1 markers (iNOS and CXCL10) were suppressed and M2 markers (Arginase1, MR, Ym1 and Trem2) were increased in a polyp size-dependent manner. Data represent fold induction of mRNA expression compared with histologically normal mucosa (ctrl) ($n = 8$). $^*P < 0.05$. (**E**) Polyps were double stained with F4/80 and iNOS (M1 marker, showing negative staining) or F4/80 and MR (M2 marker, showing positive colocalization). Original magnification, ×400.

Statistics

All values are presented as mean ± SD unless otherwise stated. Two-tailed Student's *t*-test was used for statistical analysis. A P value <0.05 was considered significant.

Results

Stroma of $Apc^{Min/+}$ mouse polyps was infiltrated by TAMs that were polarized to M2 phenotypes

We first investigated the status of TAMs in intestinal polyps of $Apc^{Min/+}$ mice. Here, we defined polyps with 1–2 mm in diameter as 'small' polyps and those >2 mm in diameter as 'large' polyps. Immunohistochemistry showed that polyp stroma was infiltrated by a massive number of F4/80-positive macrophages (Figure 1A and B). Although macrophages are a major stromal component in histologically normal mucosa of the small intestine, messenger RNA (mRNA) expression of F4/80 in polyps was increased in a size-dependent manner (Figure 1C). This finding was consistent with previous reports demonstrating massive infiltration of TAMs in mouse intestinal tumor stroma (28).

To evaluate the phenotype of TAMs in $Apc^{Min/+}$ mouse polyps, we next investigated mRNA expression of representative M1 and M2 genes by qRT–PCR (Figure 1D). mRNA of iNOS and C-X-C motif chemokine 10 (CXCL10), which are key effector molecules produced by M1 macrophages, were significantly lower in small and large polyps compared with histologically normal mucosa. In contrast, mRNA of arginase 1, Ym1, MR and triggering receptor expressed on myeloid cells 2 (Trem2), which are typical M2 markers, were higher in small and large polyps. Immunohistochemistry was consistent with these findings. F4/80- and iNOS-positive macrophages were scarcely detected in polyp stroma, but a number of F4/80- and MR-positive macrophages had infiltrated the stroma (Figure 1E). Taken together, TAMs infiltrating the small intestinal polyp stroma of $Apc^{Min/+}$ mice were polarized to M2 phenotypes.

Th2 cytokines were predominant in the microenvironment of $Apc^{Min/+}$ mouse polyps

Since TAMs can alter their phenotype in response to the microenvironment in which they exist, we sought to investigate the factors that determine the M2 phenotype in TAMs in $Apc^{Min/+}$ mouse polyps. mRNA expression of IFN-γ and IL-12, which are responsible for driving Th1 responses and antitumor activity, were suppressed in polyps compared with histologically normal mucosa (Figure 2A). In contrast, mRNA of IL-4, IL-13 and IL-10, which induce M2 macrophage phenotype, were higher in polyps than in histologically normal mucosa (Figure 2B). Thus, the cytokine expression profile of $Apc^{Min/+}$ mouse polyps was consistent with the M2 TAM phenotype we observed.

COX-2 inhibitor reduced the size and number of polyps and altered TAM phenotype from M2 to M1 in $Apc^{Min/+}$ mice

Subsequently, we investigated the effect of COX-2 inhibition on TAM activation in $Apc^{Min/+}$ mouse polyps. Similar to previously reported studies (9), mRNA levels of COX-2 showed 2.01 ± 1.6 (mean ± SD)-fold elevation in small polyps as compared with histologically normal mucosa, and COX-2 protein was immunostained in polyp stroma cells, including F4/80-positive cells (Figure 3A).

To determine whether COX-2 inhibition affects TAM phenotype, we administered celecoxib, a widely used COX-2 selective inhibitor, to $Apc^{Min/+}$ mice for 8 weeks. We used 330 or 66 μg/body wt g/day of celecoxib based on previous reports (23,29). The number and size of small intestinal polyps were decreased in $Apc^{Min/+}$ mice treated with 330 or 66 μg of celecoxib (Figure 3B and C). We also used 6.6 μg of celecoxib, which is usual daily dose in human and could not find significant suppression of intestinal polyps in $Apc^{Min/+}$ mice (data not shown). As well as previous reports (10), we considered the required dose of COX-2 inhibitor would be different between mouse and human, and used 330 μg/body wt g/day of celecoxib in the following mouse studies. As described previously (10), large polyps almost disappeared in mice fed with celecoxib, we studied the phenotypes of

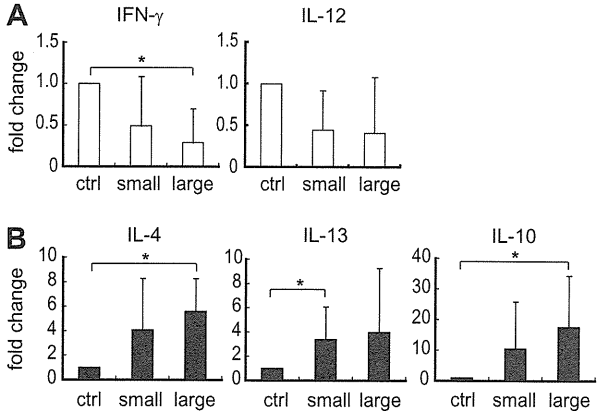

Fig. 2. $Apc^{Min/+}$ mouse polyps showed a Th2-predominant tumor microenvironment. (**A** and **B**) qRT–PCR analysis was performed for cytokine milieu in $Apc^{Min/+}$ mouse polyps. Histologically normal mucosa was used as control (ctrl) ($n = 6$). $^*P < 0.05$. (A) Th1 cytokines (IFN-γ and IL-12) were suppressed in $Apc^{Min/+}$ mouse polyps in a polyp size-dependent manner. (B) Th2 cytokines (IL-4, IL-13 and IL-10) were upregulated in $Apc^{Min/+}$ mouse polyps in a polyp size-dependent manner.

TAMs using the same size fraction of polyps 1–2 mm in diameter with or without celecoxib. As shown in Figure 3D, mRNA of the M1 genes iNOS and CXCL10 were restored with COX-2 inhibition. In contrast, mRNA of the M2 genes arginase 1, Ym1, MR and Trem2 were markedly downregulated in polyps and were almost similar to those in histologically normal mucosa with COX-2 inhibition. Thus, COX-2 inhibition skewed TAM phenotype from M2 to M1 in $Apc^{Min/+}$ mouse polyps.

COX-2 inhibition resulted in significant upregulation of IFN-γ in $Apc^{Min/+}$ mouse polyps

Based on the alteration of TAM phenotypes, we presumed that COX-2 inhibition may also alter Th1/2 cytokine levels in Apc^{Min} mouse polyps. Therefore, we investigated cytokine expression in the same size fraction of polyps (1–2 mm in diameter) with or without COX-2 inhibition. Among Th1 cytokines, mRNA expression of IFN-γ was significantly higher in polyps with COX-2 inhibition than in control polyps without COX-2 inhibition (Figure 4A). On the other hand, Th2 cytokines such as IL-4, IL-13 and IL-10 were suppressed by COX-2 inhibition. However, this suppression of Th2 cytokines was not statistically significant, and therefore the Th2 cytokines appeared to remain in Apc^{Min} mouse polyps even in the presence of COX-2 inhibitor. We next examined the source of IFN-γ in Apc^{Min} mouse polyps induced by COX-2 inhibition. qRT–PCR showed mRNA expression of CD4, CD8 and Nkg2d were enhanced by COX-2 inhibition, suggesting influx of T cells and natural killer cells was increased (Figure 4B). Immunofluorescence stainings demonstrated IFN-γ expression colocalized with both CD3ε-positive T cells and Ncam-positive natural killer cells in Apc^{Min} mouse polyps with COX-2 inhibition (Figure 4C). Collectively, these findings indicated that COX-2 inhibition altered cytokine profile by enhancing T cells and natural killer cells to produce IFN-γ and might skewed M2 TAMs to M1 phenotype in tumor microenvironment.

COX-2 inhibition alone was not sufficient to alter the activation status of mouse peritoneal macrophages in the presence of IL-4, IL-13 and IL-10

In vivo studies raised a question whether up-regulation of Th1 cytokine IFN-γ by COX-2 inhibition could alter TAM phenotypes from M2 to M1 even in the presence of Th2 cytokines such as IL-4, IL-13 and IL-10. To answer this question, we examined the direct effect of these cytokines on freshly isolated mouse peritoneal macrophages in terms of M1/M2 phenotype.

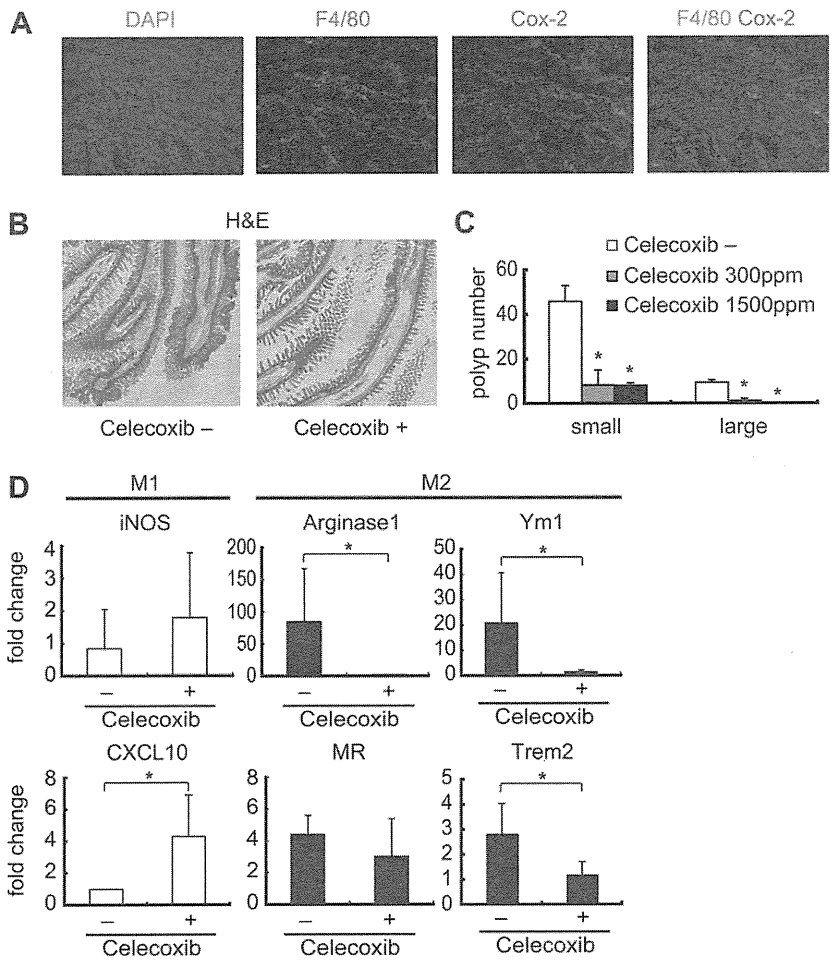

Fig. 3. COX-2 inhibitor altered TAM phenotypes from M2 to M1 in $Apc^{Min/+}$ mouse polyps. (**A**) Double staining with F4/80 (green) and COX-2 (red) showed that macrophages produced COX-2 in $Apc^{Min/+}$ mouse polyps. Original magnification, ×400. (**B**) Male $Apc^{Min/+}$ mouse were treated with either drug-free chow or 330 μg/body wt g/day celecoxib-mixed chow for 8 weeks ($n = 8$). Representative hematoxylin and eosin sections showed reduction of small intestinal polyps with celecoxib. Original magnification, ×40. (**C**) The number of small or large polyps of small intestine in control and 330 or 66 μg/body wt g/day celecoxib-treated cohorts was counted. (**D**) mRNA expression in polyps of control and celecoxib-treated cohorts was evaluated by qRT–PCR. M1 markers (iNOS and CXCL10) were upregulated and M2 markers (Arginase1, MR, Ym1 and Trem2) were downregulated. Data represent fold induction of mRNA expression compared with mRNA in control $Apc^{Min/+}$ mouse polyps ($n = 4$–8). $^*P < 0.05$.

As a previous report showed that macrophages were polarized to the M2 phenotype with IL-4 and IL-13 (4,30), we first administered 20 ng/ml of recombinant IL-4 and IL-13 to mouse macrophages. We found that the mRNA expression of the M1 marker iNOS, CXCL10 and IL-12 remained unchanged (Figure 5A). In contrast, the M2 marker MR, Trem2 and Ym1 mRNA were significantly upregulated. Thus, mouse peritoneal macrophages, though not completely, polarized to the M2 phenotype in the presence of IL-4 and IL-13, and therefore these cells seemed a model for TAMs.

To test the role of COX-2 inhibition in macrophage phenotype, we administered 10 μM celecoxib to the medium together with 20 ng/ml of recombinant IL-4 and IL-13 (Figure 5A). mRNA expression of CXCL10 was upregulated. However, mRNA of iNOS and IL-12 were not altered significantly. Thus, at the cellular levels COX-2 inhibition alone was not sufficient to alter an M2 phenotype of macrophages in the presence of IL-4 and IL-13. Because IL-4 and IL-13 induce M2a or wound healing macrophages, and IL-10 induces M2c or regulatory macrophages (5,7), we investigated the effect of recombinant IL-10 on mouse macrophages. 20 ng/ml of IL-10 also upregulated mRNA of M2 markers, MR, Trem2 and Ym1 (Figure 5B). However, administration of COX-2 inhibitor together with 20 ng/ml of IL-10 failed to alter macrophages from M2 phenotype to M1. We tested another macrophage models using human monocyte cell line THP-1 and obtained similar results to mouse macrophages (data not shown). Collectively, these results indicated that COX-2 inhibition alone was not sufficient to alter macrophage activation status from M2 to M1 in the presence of Th2 cytokines.

M2-polarized mouse macrophages were oriented to the M1 phenotype by the addition of IFN-γ even in the presence of IL-4, IL-13 and IL-10

Based on the Apc^{Min} mouse polyp data, we sought to examine the direct effect of IFN-γ on the regulation of macrophage phenotypes. Several reports demonstrate that IFN-γ prevents tumor development and reverts the M2 phenotype of TAMs (31–33). Since IFN-γ appeared to be produced by T cells and natural killer cells in Apc^{Min} mouse polyps (Figure 4B and C) and mRNA of IFN-γ in mouse macrophages was not affected by COX-2 inhibition (data not shown), we added 2, 20 or 200 ng/ml of recombinant IFN-γ to mouse peritoneal macrophages. We found that 20 ng/ml of recombinant IFN-γ significantly elevated mRNA expression of iNOS, CXCL10 and IL-12 in macrophages that had been administered 20 ng/ml of recombinant IL-4 and IL-13 (Figure 6A). In contrast, IL-4 + IL-13-induced mRNA of MR, Trem2 and Ym1 were significantly suppressed by IFN-

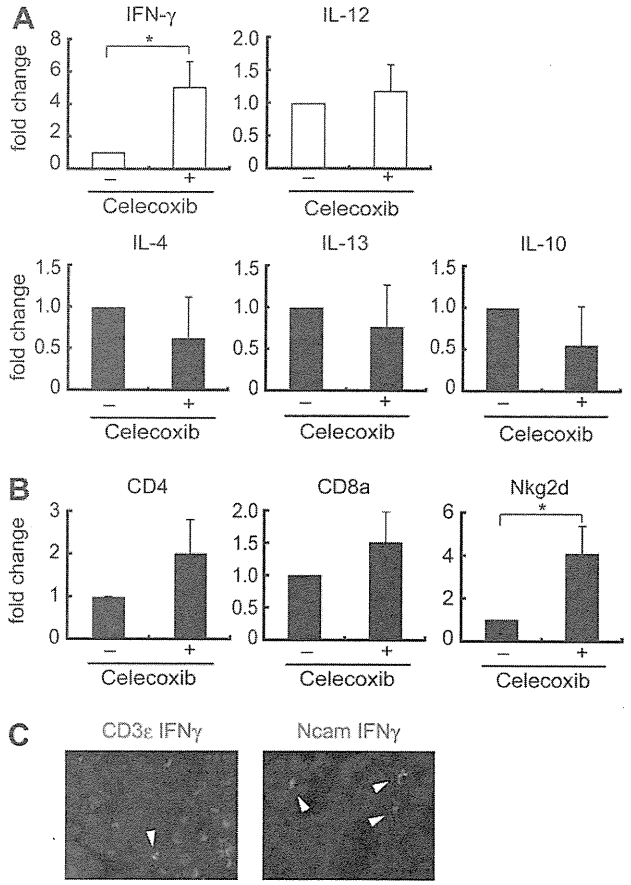

Fig. 4. Cytokine milieu in $Apc^{Min/+}$ mouse polyps showed upregulation of IFN-γ with celecoxib. (**A**) qRT–PCR analyses were performed for cytokine milieu in $Apc^{Min/+}$ mouse polyps treated with celecoxib. Data represent fold induction of mRNA expression compared with mRNA in control $Apc^{Min/+}$ mouse polyps (n = 4–8). $^*P < 0.05$ (upper). Among Th1 cytokines, the expression of IFN-γ was significantly upregulated by COX-2 inhibition (lower). Th2 cytokines (IL-4, IL-13 and IL-10) were suppressed, although not significantly, by COX-2 inhibition. (**B**) mRNA expression of CD4, CD8a and Nkg2d were elevated in 330 μg/body wt g/day celecoxib-treated $Apc^{Min/+}$ mouse polyps (n = 4–8) (**C**) IFN-γ expression (red) colocalized with both CD3 (left, green) and Ncam (right, green) in Apc^{Min} mouse polyps with COX-2 inhibition.

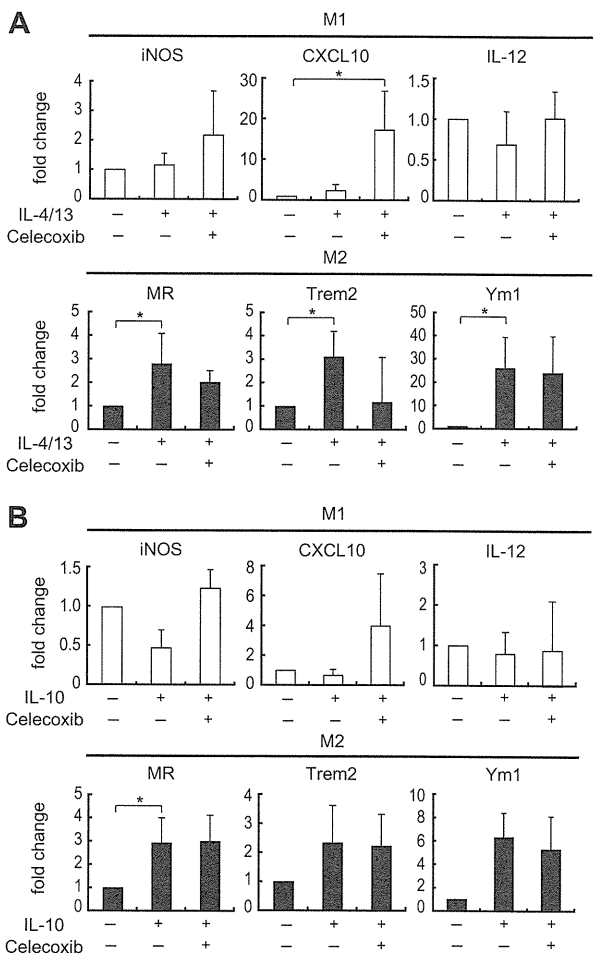

Fig. 5. COX-2 inhibition alone was not sufficient to regulate the activation status of mouse peritoneal macrophages in the presence of IL-4, IL-13 and IL-10. (**A** and **B**) mRNA expression of M1 and M2 genes was evaluated by qRT–PCR in freshly isolated mouse peritoneal macrophages. (A) When treated with 20 ng/ml of recombinant IL-4 and IL-13 for 4 h, mRNA of MR, Trem2 and Ym1 were upregulated, suggesting polarization to the M2 phenotype. Administration of 10 μM celecoxib for 4 h upregulated CXCL10. However, other markers were not altered siginificantly. $^*P < 0.05$. (B) When treated with 20 ng/ml of recombinant IL-10 for 4 h, mRNA of MR, Trem2 and Ym1 were upregulated, suggesting that macrophages were oriented to the M2 as well as IL-4 + IL-13. Administration of 10 μM celecoxib for 4 h altered M1/M2 markers in part (iNOS and CXCL10) without significant alterations. $^*P < 0.05$.

γ. Similar outcomes were obtained when the experiment was performed with 2 or 200 ng/ml of IFN-γ (data not shown). These results indicate that IFN-γ has sufficient capacity to alter the activation status of macrophages from M2 to M1 even in the presence of IL-4 and IL-13. We also confirmed the crucial role of IFN-γ on mouse macrophages in the presence of IL-10. Treatment with 20 ng/ml of IFN-γ upregulated M1 markers (iNOS, CXCL10 and IL-12) and downregulated M2 markers (MR, Trem2 and Ym1) again in mouse macrophages in the presence of 20 ng/ml of IL-10 (Figure 6B).

Discussion

In the present study, we demonstrated that TAMs in $Apc^{Min/+}$ mouse polyps possessed alternatively activated (M2) phenotypes and that the cytokine milieu of the polyps was Th2 predominant. Our *in vivo* study revealed that COX-2 inhibition altered TAM phenotypes from M2 to M1 in association with the increase of IFN-γ in the polyps, and our *in vitro* study showed that IFN-γ played a key role in the alteration of macrophage phenotypes even in the presence of the Th2 cytokines IL-4, IL-13 and IL-10. These data suggest that COX-2 inhibition alters TAM phenotype in an IFN-γ-dependent manner.

To the best of our knowledge, this study is the first direct demonstration that TAM phenotype is polarized to M2 in $Apc^{Min/+}$ mouse intestinal polyps (18). Due to the functional plasticity of macrophages, TAMs are strongly influenced by tumor microenvironment (e.g. Th1/Th2 balance) and are considered to be alternatively activated in most tumors (4,8,34–38). TAMs, especially M2 TAMs, promote tumor progression in a number of experimental models (8, 34–39). However, the significance of TAMs in gut tumorigenesis has been unclear, with some reports showing a correlation between increased number of TAMs and good prognosis, which is in contrast to other malignancies (40,41). To date, few experimental reports have directly demonstrated the role of TAMs in intestinal tumorigenesis in this context. Our study using $Apc^{Min/+}$ mouse supports the possibility