

Original

Deficiency of Macrophage Colony-Stimulating Factor Attenuates DSS-Induced Colitis in Mice

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Abstract: The purpose of this study was to investigate the role of macrophage colony-stimulating factor (M-CSF)-induced macrophages (M ϕ s) in dextran sulfate sodium (DSS)-induced colitis. M-CSF deficient (op/op) mice and their control mice were administered with DSS in drinking water for 7 days, and then colonic specimens were harvested. Histopathological findings were evaluated, and distribution of M ϕ s in the colon was assessed. Moreover, protein concentration of inflammatory cytokines in the colon was determined. In another set of experiments, isolated M ϕ s from the colonic lamina propria were stimulated with lipopolysaccharide, and then cytokine production was measured. After treatment with DSS, control mice developed severe colitis, and the number of M ϕ s increased in the colon. In op/op mice, the number of M ϕ s was reduced, and colitis was inhibited. The protein concentrations of IFN- γ and IL-12 in the colon were increased in control mice but not in op/op mice after treatment with DSS. Furthermore, production of IL-12 by isolated M ϕ s was increased in control mice but not in op/op mice. Taken together, these results suggested that M-CSF-induced M ϕ s are most likely involved in the pathogenesis of inflammatory bowel diseases.

Key words: Macrophages, Osteopetrotic mouse, Interferon gamma, Interleukin-12, Inflammatory bowel disease

Abbreviations: CD, Crohn's disease; DSS, dextran sulfate sodium; ELISA, enzyme linked immunosorbent assay; GM-CSF, granulocyte/macrophage colony-stimulating factor; GM-M ϕ , GM-CSF-induced macrophage; IBDs, inflammatory bowel diseases; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; M ϕ s, macrophages; M-CSF, macrophage colony stimulating factor; M-M ϕ , M-CSF-induced macrophage; op/op, osteopetrotic mouse; Th1, T helper 1; UC, ulcerative colitis

INTRODUCTION

Inflammatory bowel diseases (IBDs), which include ulcerative colitis (UC) and Crohn's disease (CD), represent a group of chronic disor-

ders characterized by inflammation of the gastrointestinal tract, typically with a relapsing and remitting clinical course^{1,2)}. Infiltrating activated macrophages (M ϕ s) are increased in the inflamed gut in patients with IBDs³⁾. Activated M ϕ s are thought to be major contributors to the production of inflammatory cytokines in the gut, and an imbalance of these cytokines is involved in the pathogenesis of IBDs²⁾.

Several growth factors, such as interleukin (IL)-3, granulocyte/macrophage colony-stimu-

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lating factor (GM-CSF), and macrophage colony-stimulating factor (M-CSF), are known to act on development and differentiation of specific cell lineages. It is thought that GM-CSF and IL-3 act earlier, whereas M-CSF acts later in the development and differentiation of M ϕ s⁴. M-CSF is important in the regulation of M ϕ maturation, activation, and migration. M ϕ s are divided into two subsets based on their maturation, M-CSF-induced M ϕ s (M-M ϕ s) and GM-CSF-induced M ϕ s (GM-M ϕ s), and their functions are different^{5,6}. For example, M-CSF is abundantly present *in vivo* (serum concentrations; 362–515 $\mu\text{g/ml}$), whereas GM-CSF is little present (serum concentrations; less than 2 $\mu\text{g/ml}$)⁷. The predominant source of circulating M-CSF is thought to be vascular endothelial cells, but a range of other cell types, including fibroblasts, osteoblasts, monocytes, B cells, T cells, and bone marrow stromal cells, also produce M-CSF⁷. Biologically active M-CSF is absent in the osteopetrotic (op/op) mouse, which is characterized by an autosomal recessive inactivating mutation in the M-CSF gene⁸⁻¹⁰. The number of colonic macrophages is decreased to 20% of the number in the phenotypically normal control mice⁹. Furthermore, M-CSF-independent M ϕ s in op/op mice are morphologically small and round and show ultrastructural immaturity¹⁰.

Animal models of intestinal inflammation are indispensable in understanding the pathogenesis of IBDs. Administration of DSS in drinking water induces colitis characterized by bloody diarrhea, ulcerations and infiltrations with granulocytes^{11,12}. It is generally believed that DSS is directly toxic to gut epithelial cells of the basal crypts and affects the integrity of the mucosal barrier¹¹. On the other hand, it was reported that tissue-specific M ϕ s in the colon are also involved in the mechanism of this colitis

model¹¹. Since intestinal tissues predominantly express M-CSF⁶, the specific purpose of this study was to investigate the role of M-M ϕ s in DSS-induced colitis.

MATERIALS AND METHODS

Animals and treatments

M-CSF-deficient mice (B6C3Fea/a-CSFop/J, op/op) and control mice (op/?) were purchased from Jackson Laboratories (Bar Harbor, ME). Male mice (8 weeks of age) were used in this experiment (n=8 in each group). There was no significant difference in the mean body weight before experiments between the two groups (op/op mice, 20.1 \pm 1.1 g; and control mice, 20.0 \pm 1.2 g). All animal experiments were approved by University of Yamanashi Animal Care and Use Committee. The experimental protocol followed the institutional and the National Research Council criteria for the care and use of laboratory animals in research. Furthermore, all mice received humane care in compliance with institutional guidelines. Mice were given 3% DSS (molecular weight of 5000; Wako Pure Chemical Industries, Ltd.) orally in drinking water for 7 days^{11,12}. After administration of DSS, mice were given distilled water for 7 days. Severity of disease was assessed using the following clinical disease activity score: 1 = appearance of diarrhea, 2 = signs of fecal blood, 4 = profuse bleeding from the anus².

Histological measurement

Sections of the colon stained with hematoxylin-eosin were assessed in a blinded fashion using a scoring system¹⁴. These changes were also quantified for the percentage involvement by the disease process: (1) 1–25%; (2) 26–50%; (3) 51–75%; (4) 76–100%. Each section was then scored for each feature separately by estab-

lishing the product of the grade for that feature and the percentage involvement (in a range from 0 to 12 for inflammation and extent, and in a range from 0 to 16 for crypt damage).

Immunohistochemistry

Cryopreserved 4 μm -thick sections were fixed in cold acetone and washed in tris-phosphate buffer (TBS). Slides were then incubated in 1% bovine serum albumin for 60 min. Distribution of M ϕ s was detected by F4/80 antibody (Clone: A3-I Serotec, Oxford, England), and expression of IL-18 was detected by anti-mouse IL-18 antibody (Santa Cruz, CA). Primary antibody was added at a dilution of 1:50 and incubated overnight at room temperature, then further processed for immunohistochemistry using a vectastain ABC kit (Vector Laboratories, Burlingame, CA). Peroxidase activity was developed in diaminobenzidine as a chromogen^{13, 15}.

Cytokine concentration in the colon

The colonic tissue was homogenized in cold phosphate-buffered saline using a Polytron-type homogenizer. Tissue homogenate was then centrifuged at 20,000 \times rpm for 20 mins at 4°C to obtain the supernatant. Total protein concentrations of the tissue supernatant were measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) for calibration, and protein concentrations of cytokines in the tissue homogenate were determined using enzyme-linked immunosorbent assay (ELISA) kits (TNF- α , IFN- γ , IL-1 β , IL-6, IL-12, IL-17, IL-23: R&D Systems, Minneapolis, MN; IL-18: Immune-Biological Laboratories, Gunma, Japan) according to the manufacturer's instructions¹⁶.

Isolation of colonic lamina propria macrophages.

Lamina propria mononuclear cells were iso-

lated using a protocol described by Kamada *et al*⁶). Briefly, isolated colons were washed with HBSS, dissected into small pieces, and incubated in HBSS containing 2.5% FBS and 1 mM DTT (Sigma-Aldrich, St. Louis, MO) to remove any mucus. The pieces were then incubated in HBSS containing 1 mM EDTA (Sigma-Aldrich) twice for 20 min each at 37°C, and incubated in HBSS containing 1 mM collagenase type IV (Sigma-Aldrich) for 2 hr at 37°C. Isolated cells were resuspended in 40% Percoll (GE Healthcare, Chicago, IL), layered onto 75% Percoll, and centrifuged at 2,000 rpm for 20 min. Cells were recovered from the interphase and washed with PBS. Cells were seeded onto 25-mm glass coverslips and cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO Laboratories Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 10 mmol/L HEPES, and antibiotics (100 U/mL penicillin G and 100 mg/mL streptomycin sulfate) at 37°C with 5% CO₂. Nonadherent cells were removed after 1 hr by replacing the culture medium. All adherent cells phagocytosed latex beads, indicating that they were M ϕ s. Cells were cultured for 24 hr and used for measurement of cytokine production¹⁷.

Cytokine production by isolated macrophages.

M ϕ s isolated from op/op mice or control mice were seeded onto 24-well plates and cultured in DMEM supplemented with 10% FBS and antibiotics at 37°C in the presence of 5% CO₂. After 24 hr of incubation, cells were incubated with fresh media containing LPS (10 $\mu\text{g}/\text{mL}$) supplemented with 5% mouse serum for an additional 4 hr. Media were collected and kept at -80°C until assay. TNF- α , IL-1 β , IL-6, IL-12, IL-18 and IL-23 in the culture media were measured using an ELISA kit and data were corrected for dilution¹⁷.

Statistical Analysis

Data are expressed as mean \pm SE. Analysis of variance (ANOVA) with Bonferroni's post hoc test or the Student's t-test was used to determine significance when appropriate. A p value less than 0.05 was considered significant.

RESULTS

Clinical findings

During the experiment, there were no significant differences between 2 groups in the total amount of DSS consumed. After administration of DSS, significant body weight loss was seen in control mice. On the other hand, body weight did not change in op/op mice throughout the experimental period (data not shown). In control mice following administration of DSS, diarrhea was seen after 3 days, melena was seen after 5 days, and profuse bleeding from the anus was seen after 7 days. In contrast, there was no sign of bleeding in op/op mice during the experimental period. Moreover, the disease activity index was significantly lower in op/op mice compared with control mice (Fig. 1A).

Histopathological findings

Mice given distilled water did not show any pathological changes in the colon (Figs. 1B, 1C). However, severe inflammatory cell infiltration, complete crypt loss and destruction of the epithelium were seen in control mice treated with DSS (Fig. 1D). In contrast, inflammatory cell infiltration and shortening of crypts were prevented in op/op mice (Fig. 1E). Pathological scores were significantly lower in op/op mice compared with control mice (Table 1).

Immunohistochemistry for F4/80

The distribution of M ϕ s was assessed by immunohistochemistry using F4/80. The number

of M ϕ s was significantly lower in op/op mice compared with control mice before treatment with DSS (Figs. 2A and 2B). After treatment with DSS, the number of M ϕ s was increased

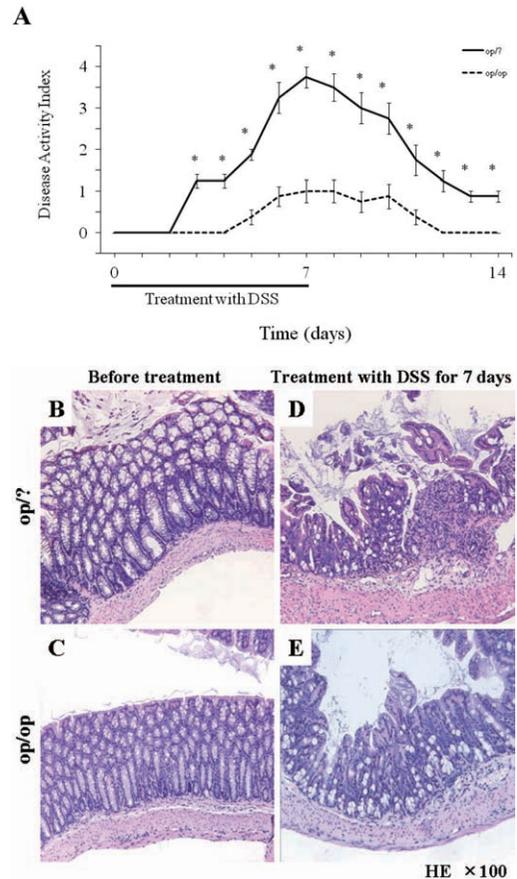


Fig. 1. Disease activity index and pathological findings in the colon after DSS treatment.

Disease activity index (A) was calculated as described in materials and methods. Representative photomicrographs. Data represents mean \pm SE (n=8). op/?, control mice; and op/op, op/op mice. *, P < 0.05 compared with op/op mice by ANOVA with Bonferroni's post hoc test. Representative microphotographs of colonic tissues from control mouse before treatment (B); op/op mouse before treatment (C); control mouse treated with DSS for 7 days (D); and op/op mouse treated with DSS for 7 days (E). Original magnification, \times 100. op/?, control mouse; and op/op, op/op mouse.

Table 1. Pathological score in the colon after treatment with DSS for 7 days

		Proximal colon	Distal colon
Inflammation	op/?	6.75 ± 1.24	7.00 ± 1.25
	op/op	2.50 ± 0.57*	3.00 ± 0.46*
Extent of inflammation	op/?	5.00 ± 0.85	7.75 ± 0.25
	op/op	1.75 ± 0.49*	2.75 ± 0.49*
Crypt damage	op/?	7.50 ± 2.03	12.00 ± 1.07
	op/op	4.25 ± 1.61*	3.75 ± 0.56*
Pathological score	op/?	19.25 ± 4.05	26.75 ± 2.10
	op/op	8.50 ± 2.51*	9.50 ± 1.21*

Pathological score was calculated as described in materials and methods. Data represent mean ± SE (n = 8). op/?, littermate mice; and op/op, op/op mice. *, p < 0.05 compared with littermate mice.

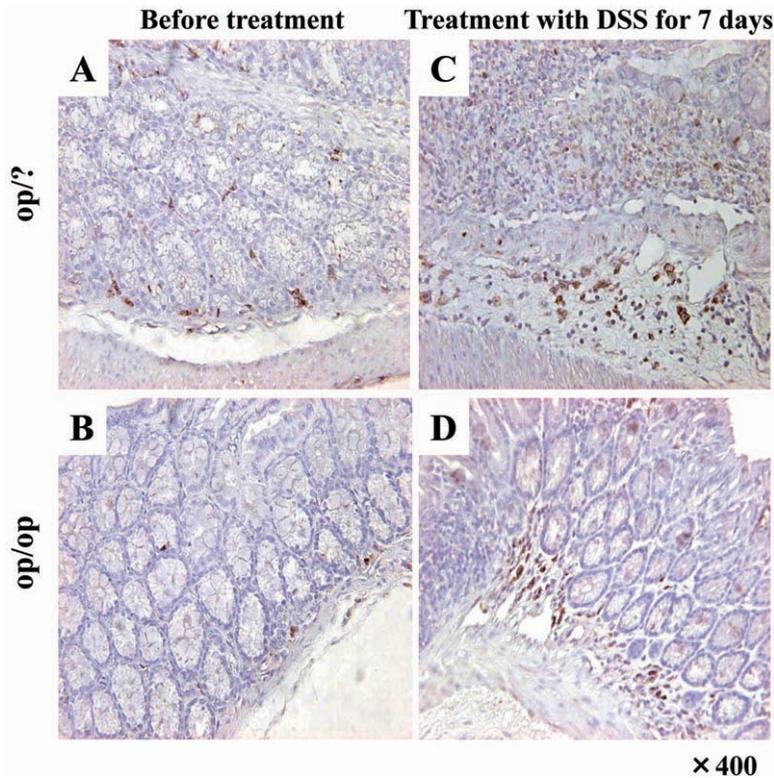


Fig. 2. Immunohistochemistry for F4/80.

Distribution of Mφs in the colon was assessed by immunohistochemistry for F4/80. Representative photomicrographs are shown. Original magnification, × 400. A, control mouse before treatment; B, op/op mouse before treatment; C, control mouse treated with DSS for 7 days; D, op/op mouse treated with DSS for 7 days. op/?, control mouse; and op/op, op/op mouse.

markedly in control mice (Fig. 2C). On the other hand, the number of M ϕ s was less in op/op mice compared with control mice (Fig. 2D).

Immunohistochemistry for IL-18

Before treatment with DSS, IL-18-positive cells were mainly detected in the epithelium, and fewer mononuclear cells were present in the lamina propria (Figs. 3A, 3B). There were no difference differences between two groups. After treatment with DSS, IL-18-positive mononuclear cells were increased in the inflammato-

ry region in both groups (Figs. 3C and 3D). In control mice, IL-18-positive epithelial cells were decreased compared with mice before the treatment, but not in op/op mice (Figs. 3C and 3D).

Cytokine levels in the colonic tissue

In the normal colonic tissue, protein levels of IFN- γ , IL-12, and IL-18 were minimal, and there were no significant differences between op/op mice and control mice. After administration of DSS, IFN- γ protein levels were increased significantly in control mice being about 3-fold greater compared with op/op mice at day 7 after DSS

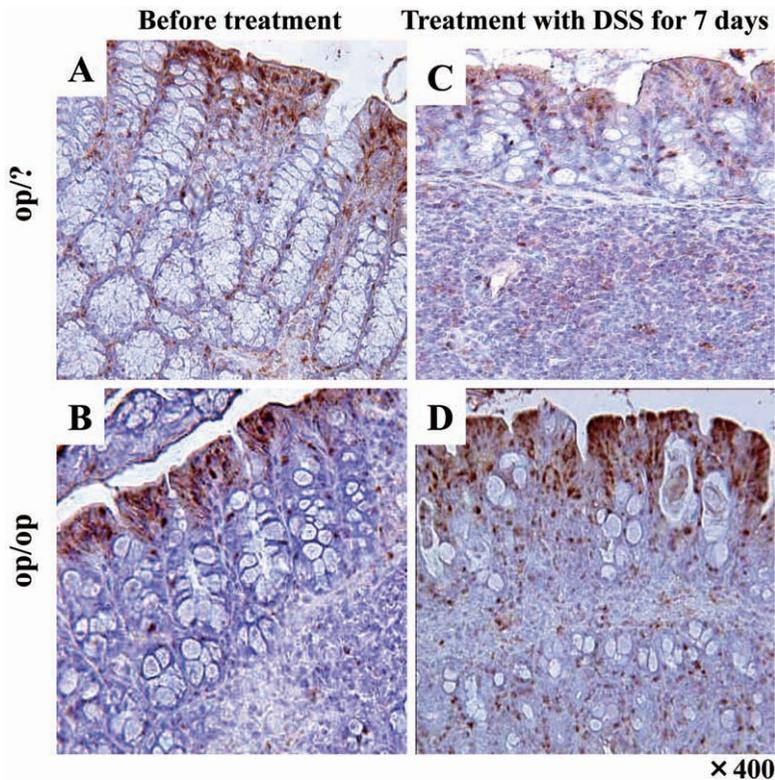


Fig. 3. Immunohistochemistry for IL-18. Expression of IL-18 in the colon was assessed by immunohistochemistry. Representative photomicrographs are shown. Original magnification, $\times 400$. A, control mouse before treatment; B, op/op mouse before treatment; C, control mouse treated with DSS for 7 days; D, op/op mouse treated with DSS for 7 days. op/?, control mouse; and op/op, op/op mouse.

treatment (Fig. 4A). Protein levels of IL-12 were also elevated significantly in control mice (Fig. 4B). On the other hand, the levels of IL-12 did not increase in op/op mice (Fig. 4B). Furthermore, protein levels of IL-18 were elevated significantly in both groups. Interestingly, concentrations of IL-18 were about 2-fold greater in op/op mice compared with control mice (Fig. 4C). Protein levels of IL-23 and IL-17 did not increase in either groups (data not shown). Levels of TNF- α , IL-1 β , and IL-6 in the colon were significantly lower in op/op mice compared with control mice after treatment with DSS (data not shown).

Cytokine production by isolated M ϕ s from the colonic lamina propria.

Production of IL-12 and IL-18 by isolated M ϕ s from the colonic lamina propria was minimal before stimulation with LPS (Fig. 5). In contrast, IL-12 production by isolated M ϕ s was increased markedly by stimulation with LPS in control mice, but not in op/op mice (Fig. 5A). In both groups, IL-18 production increased by stimulation with LPS (Figs. 5B). Production of TNF- α , IL-1 β and IL-6 slightly increased by stimulation with LPS, and there were no significant difference between two groups (data not shown). Furthermore, IL-23 production did not increase by stimulation with LPS in either group (data not shown).

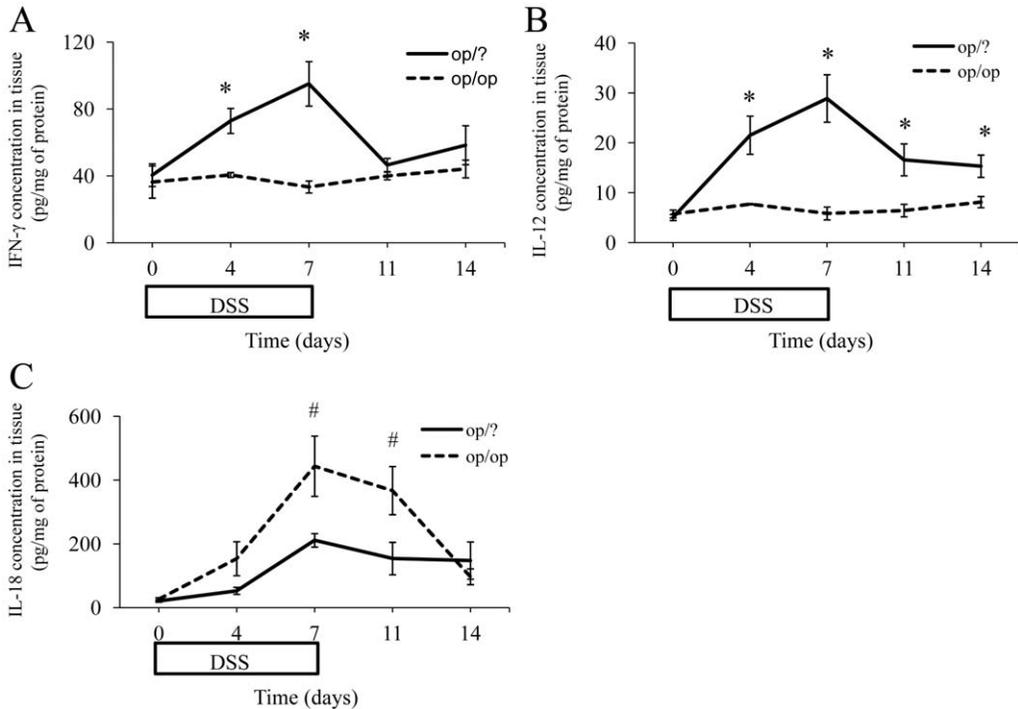


Fig. 4. Protein concentration of IFN- γ , IL-12, and IL-18 in the colon after treatment of DSS. Protein concentration of cytokines in the colon was measured by ELISA. Data represents mean \pm SE (n=8). op/?, control mice; and op/op, op/op mice. #, $p < 0.05$ compared with control mice by ANOVA with Bonferroni's post hoc test. *, $p < 0.05$ compared with op/op mice by ANOVA with Bonferroni's post hoc test.

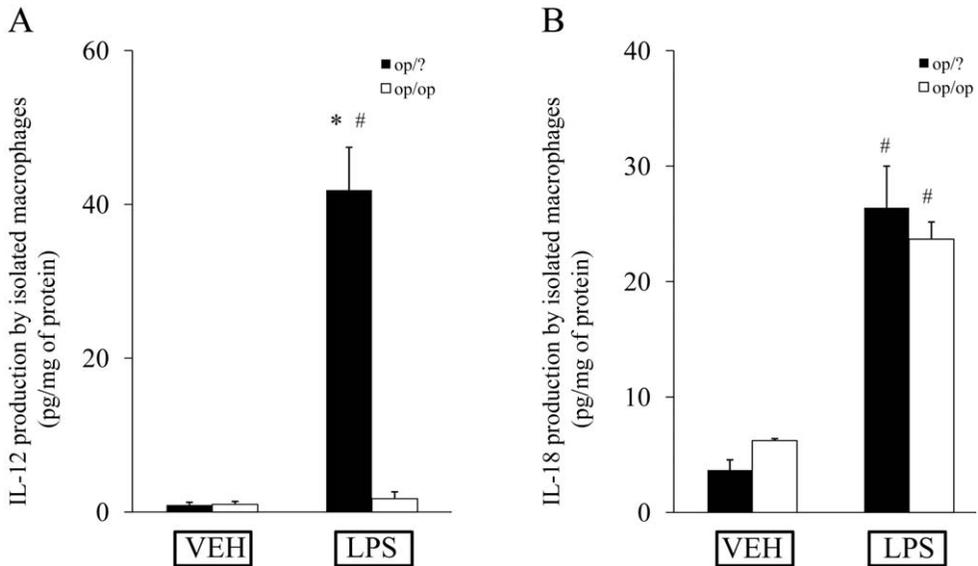


Fig. 5. Cytokine production by isolated M ϕ s from the colon. Isolated M ϕ s from the colon were stimulated with LPS (10 μ g/ml) for 4 hr. Concentration of cytokines in the supernatant was measured by ELISA. Data represents mean \pm SE (n=8). VEH, a saline vehicle; op/?, control mice; and op/op, op/op mice. #, $p < 0.05$ compared with M ϕ s isolated from control mice without LPS stimulation by ANOVA with Bonferroni's post hoc test. *, $p < 0.05$ compared with M ϕ s isolated from op/op mice with LPS stimulation by ANOVA with Bonferroni's post hoc test.

DISCUSSION

Mucosal M ϕ s play an important role in the mucosal immune system, and M-CSF regulates proliferation, differentiation, and activation of M ϕ s⁴. Op/op mice lack M-CSF, and have fewer M ϕ s, suggesting that M-M ϕ might be absent. In the present study, DSS-induced colitis was blunted in op/op mice compared with control mice (Table 1, Figs. 1, 2), leading to the conclusion that M-CSF and M-M ϕ contribute to inflammation in DSS-induced colitis in mice. In the active phase of IBDs, expression of M-CSF also increases in the colon¹⁸. Therefore, these phenomena suggest that M-CSF and M-M ϕ are possibly involved in the pathogenesis of IBDs.

T helper 1 (Th1) immunity plays an important role in the pathogenesis of IBDs^{19,20}. IL-12, which is predominantly produced by M ϕ s,

induces IFN- γ synthesis and promotes Th1 cell differentiation¹⁹. In the present study, protein levels of IFN- γ and IL-12 in the colon were markedly increased, and severe colitis were seen in control mice after treatment with DSS (Figs. 1, 4A, 4B). In contrast, protein levels of IFN- γ and IL-12 in the colon were minimal, and colitis was blunted in op/op mice (Figs. 1, 4A, 4B). These findings indicate that increased expression of IFN- γ and IL-12 in the colon most likely contributes to pathogenesis of colitis in this experimental model.

IL-18 is mainly produced by T cells, B cells, M ϕ s and epithelial cells^{21,22}. IL-18 acts synergically with IL-12 in production of IFN- γ and involved in the pathogenesis of IBDs^{23,24}. In contrast, it was also reported that IL-18 knockout mice developed more severe colitis compared with wild-type mice after treatment with DSS²⁵. Fur-

thermore, IL-18 promotes wound healing in the epithelium^{26,27}. In the present study, protein levels of IL-18 in the colon were significantly greater, and colitis was blunted in op/op mice compared with control mice (Figs 1, 4C). Furthermore, the DSS treatment decreased IL-18-positive epithelial cells (Fig. 3D) and delayed regeneration of the epithelium in control mice (data not shown). These phenomena indicate that prolonged inflammation affects production of IL-18 by the epithelium and suppresses regeneration of the epithelium in the colon.

M ϕ s produce inflammatory cytokines such as TNF- α , IL-1 β and IL-6^{28,29}. In the present study, protein levels of TNF- α , IL-1 β and IL-6 in the colon were increased significantly in control mice compared with op/op mice as previously described by Ghia *et al*¹³. (data not shown). In contrast, there were no differences in production of TNF- α , IL-1 β , and IL-6 by isolated colonic M ϕ s after stimulation with LPS (data not shown), suggesting that two subsets of M ϕ s have same ability to produce TNF- α , IL-1 β and IL-6. Thus, major source of these cytokines might be inflammatory cells such as lymphocyte or granulocytes.

Since op/op mice lack M-CSF, the population of colonic M ϕ s in op/op mice are predominantly GM-M ϕ s. Production of IL-12 by isolated M ϕ s increased after LPS stimulation in control mice but not in op/op mice (Fig. 5A). This finding supports the hypothesis that M-M ϕ -derived IL-12 induces production of IFN- γ from T cells, which induces inflammation in the colon.

CONCLUSION

M-CSF deficiency reduced IL-12 production by isolated colonic M ϕ s and decreased inflammation in DSS-induced colitis. Blocking M-CSF and activity of M-M ϕ s may become a new thera-

peutic target for IBDs.

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