Role of metallothionein in murine experimental colitis

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Received November 15, 2012; Accepted January 7, 2013

DOI: 10.3892/ijmm.2013.1294

Abstract. Metallothioneins (MTs) are a family of cysteine-rich low molecular-weight proteins that can act as reactive oxygen species scavengers. Although it is known that the induction of MT expression suppresses various inflammatory disorders, the role of MTs in intestinal inflammation remains unclear. In this study, we investigated the effects of dextran sulfate sodium (DSS) administration in mice with targeted deletions of the MT-I/II genes. Acute colitis was induced by 2% DSS in male MT-I/II double knockout (MT-null) and C57BL/6 (wild-type) mice. The disease activity index (DAI) was determined on a daily basis for each animal, and consisted of a calculated score based on changes in body weight, stool consistency and intestinal bleeding. Histology, colon length, myeloperoxidase (MPO) activity and colonic mRNA expression and the concentration of inflammatory cytokines were evaluated by real-time-PCR and enzyme-linked immunosorbent assay (ELISA). The localization of MTs and macrophages was determined by immunohistological and immunofluorescence staining. To investigate the role of MTs in macrophages, peritoneal macrophages were isolated and their responses to lipopolysaccharide were measured. Following DSS administration, the DAI score increased in a time-dependent manner and was significantly enhanced in the MT-I/II knockout mice. Colonic MPO activity levels and inflammatory cytokines [tumor necrosis factor (TNF)-α, interferon (IFN)-γ and interleukin (IL)-17] production increased following DSS administration, and these increases were significantly enhanced in the MT-I/II knockout mice compared with the wild-type mice. MT-positive cells were detected in the lamina propria and submucosal layer by immunohistochemical and immunofluorescence staining, and were mainly co-localized in F4/80-positive macrophages. The production of inflammatory cytokines (TNF-α, IFN-γ and IL-17) from isolated peritoneal macrophages increased following lipopolysaccharide stimulation, and these increases were significantly enhanced in the macrophages obtained from the MT-I/II knockout mice. These data indicate that MTs play an important role in the prevention of colonic mucosal inflammation in a mouse model of DSS-induced colitis, thus suggesting that endogenous MTs play a protective role against intestinal inflammation.

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Key words: metallothioneins, dextran sulfate sodium, intestinal inflammation, macrophages

Introduction

Inflammatory bowel diseases (IBDs) such as ulcerative colitis and Crohn's disease are known as refractory and recurrent diseases of the gastrointestinal tract. Although the features of IBDs suggest a number of possible causes, including genetic, infectious and immunological factors, the precise pathogenesis of IBDs remains unknown (1-3). Recent evidence suggests that oxidative stress caused by reactive oxygen species (ROS) is an important factor involved in the onset and development of intestinal inflammation. Furthermore, it has also been demonstrated that disruptions in the antioxidant defense system are involved in the pathophysiology of IBDs (4,5). Therefore, it is important to investigate the oxidative stress-related pathogenesis of IBDs.

Metallothioneins (MT) are a family of low molecular weight proteins containing multiple cysteine residues that coordinate multiple zinc and copper atoms, enabling a high affinity for monovalent and divalent heavy metal atoms (6). The presence of MTs has been confirmed in mammals, birds, fish, amphibians, reptiles and invertebrates, as well as in a wide range of plants, and eukaryotic and prokaryotic microorganisms (7). In mice, 4 MT isoforms have been identified: MT-I and MT-II are ubiquitously expressed, MT-III is expressed predominantly in the brain and MT-IV is expressed in stratified squamous epithelial cells (8,9).

In humans, MTs are encoded by at least 10 identified functional genes, and the encoded proteins are conventionally subdivided into 4 groups: MT-I, MT-II, MT-III and MT-IV (10). As MTs have a high affinity for metals, as noted above, MTs have free radical-scavenging potential, with MT-I/II exerting particularly potent antioxidant effects in comparison with the other isoforms.

As regards the role of MTs in various pathophysiological conditions causing inflammation, MTs have been reported to play a protective role in various animal models, including lipopolysaccharide (LPS)-induced lung injury (11), rheumatoid arthritis (12), multiple sclerosis (13), coagulatory disturbance (14), ethanol-induced gastroduodenal mucosal injury (15) and *Helicobacter pylori*-induced gastritis (16). MT-deficient mice are more susceptible to LPS-induced lethal shock following D-galactosamine (D-GalN) sensitization through the reduction of α1-acid glycoprotein (17). By contrast, there are conflicting data in a tumor necrosis factor (TNF)-induced lethal shock model suggesting a reduction in mortality in MT-deficient mice, as compared to wild-type mice (18). Furthermore, MT-overexpressing mice are also more sensitive to the lethal effects of TNF compared to wild-type mice (19).

Although MTs are known to a play protective role in intestinal inflammation, studies on experimental colitis models using MT-deficient mice have revealed that MTs do not protect against the development of colitis. Tran *et al* (20) demonstrated that MT-deficient mice had significantly lower disease activity index (DAI) than wild-type mice in a dextran sodium sulfate (DSS)-induced colitis model, a widely accepted model of human IBD. Oz *et al* (19) also reported that there were no differences in histological damage following treatment with DSS among wild-type, MT-deficient and MT transgenic mice. Hence, the function of MTs in intestinal inflammation remains uncertain.

In the present study, we examined the role of MTs in intestinal inflammation using a mouse model of DSS-induced colitis, which is recognized as a useful experimental model of intestinal inflammation and is considered to be similar to human IBD (21-24).

Materials and methods

Animals. We used 7-week-old male MT-I/II double knockout (MT null) mice (25) and age-matched C57BL/6 wild-type mice. MT-I/II knockout mice were kindly provided by Dr Hirohisa Takano (Center for Environmental Health Sciences, National Institute for Environmental Studies, and Kyoto University, Graduate School of Engineering, Kyoto, Japan). C57BL/6 mice were purchased from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan). MT-I/II knockout mice were routinely bred in the *vivarium* of Kyoto Prefectural University of Medicine. Mice were housed in cages under a 12-h light/dark cycle, and a controlled temperature of 22˚C and negative atmospheric pressure. Mice were maintained in a specific pathogen-free environment and provided with tap water and food *ad libitum*. Mice were fed a rodent diet (CE-2; Nihon CLEA, Tokyo, Japan) during the experiment. All experimental procedures were carried out in accordance with the National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals. Experimental protocols were approved by the Animal Care and Use Committee of Kyoto Prefectural University of Medicine (Kyoto, Japan).

Induction of colitis by DSS. Male wild-type mice and MT-I/II knockout mice (7 weeks old) were randomized into control and experimental groups (6-8 mice in each group). Experimental acute colitis was induced by treatment with 2.0% DSS (molecular weight 1,000-9,000, Lot SDR4219; Wako Pure Chemical Industries, Osaka, Japan) in drinking water for 7 days, as reported previously (26,27). Mice were sacrificed under anesthesia 7 days after DSS treatment, and the colons were removed for macroscopic and histological examination. Colonic specimens were also obtained for biochemical assay and RNA isolation.

Evaluation of the severity of colitis. DAI, colon length and histology were analyzed. DAI was determined by scoring changes in animal weight, occult blood positivity, gross bleeding and stool consistency, as described previously (26,28,29). We used 5 grades of weight loss (0, no loss or weight gain; 1, 1-5% loss; 2, 5-10% loss; 3, 10-20% loss; 4, >20% loss), 3 grades of stool consistency (0, normal; 2, loose; and 4, diarrhea), and 3 grades of occult blood (0, negative; 2, occult blood-positive; and 4, gross bleeding) based on previous studies (26,27). After determining DAI, the mice were sacrificed, and the entire colon was removed from the cecum to the anus, and colon length was measured as an indirect marker of inflammation.

Immediately after dissection, the distal colon was fixed in 10% buffered formalin for histological analysis. Sections (4-μm-thick) were prepared and stained with hematoxylin and eosin (H&E). The slides were then examined and scored in a blinded manner using a previously published grading system (30,31). Briefly, a combined score of inflammatory cell infiltration and tissue damage was determined as follows: as regards cell infiltration: score 0, occasional inflammatory cells in the lamina propria; 1, increased inflammatory infiltrate in the lamina propria predominantly at the base of crypts; 2, confluence of inflammatory infiltrate extending into the mucosa; and 3, transmural extension of inflammatory infiltrate. As regards tissue damage: score 0, no mucosal damage; 1, partial (up to 50%) loss of crypts in large areas; 2, partial to total 50-100% loss of crypts in large areas, epithelium intact; and 3, total loss of crypts in large areas and epithelium lost. The total histological score represents the sum of the cell infiltration and tissue damage scores, and thus ranges from 0 to 6.

Measurement of myeloperoxidase (MPO) activity. MPO activity in the colonic mucosa, an index of polymorphonuclear leukocyte accumulation, was determined using a modification of the method described by Grisham *et al* (32). A total of 2 ml of mucosal homogenate was centrifuged at 20,000 x g for 15 min at 4˚C to pellet the insoluble cellular debris. The pellet was then re-homogenized in an equivalent volume of 0.5% hexadecyltrimethylammonium bromide. Samples were centrifuged at 20,000 x g for 15 min at 4˚C, and the supernatants were saved. MPO activity was assessed by measuring the H_2O_2-dependent oxidation of 3,3',5,5'-tetramethylbenzidine. One unit of enzyme activity was defined as the amount of MPO that caused the absorbance to change by 1.0/min at 655 nm and 25˚C.

RNA analysis. The mRNA expression levels of MT-I/II, TNF-α, interferon (IFN)-γ, interleukin (IL)-17 and β-actin (internal standard) were determined by real-time-PCR. Samples for mRNA isolation were removed from colonic tissue. Total RNA

was isolated with the acid guanidinium phenol chloroform (AGPC) method using Isogen reagent (Nippon Gene, Toyama, Japan). RNA was stored at 70˚C until it was used for reverse-transcription polymerase chain reaction (RT-PCR). A total of 1 μg of extracted RNA was reverse-transcribed into first-strand complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR for CINC-1 and β-actin was carried out with the 7300 Real-Time PCR system (Applied Biosystems) using the DNA-binding dye SYBR®-Green for the detection of PCR products. Primers had the following sequences: MT-I sense, 5'-GCTGTGCCTGATGTGACGAA-3' and antisense, 5'-AGGAAGACGCTGGGTTGGT-3'; MT-II sense, 5'-TGCGC TCGACCCAATACTC-3' and antisense, 5'-TCTAGGAGCG TGATGGAGAGAAG-3'; TNF-α sense, 5'-ATCCGCGACGT GGAACTG-3' and antisense, 5'-ACCGCCTGGAGTTCTG GAA-3'; IFN-γ sense, 5'-CCTGCGGCCTAGCTCTGA-3' and antisense, 5'-CCATGAGGAAGAGCTGCAAAG-3'; IL-17 sense, 5'-TCATCTGTGTCTCTGATGCTGTTG-3' and anti-sense, 5'-TCGCTGCTGCCTTCACTGT-3'; and β-actin sense, 5'-GAGCAAACATCCCCCAAAGTT-3' and antisense, 5'-GCC GTGGATACTTGGAGTGACT-3'. Relative quantities of gene expression with real-time PCR data were calculated relative to β-actin.

Determination of colonic concentrations of TNF-α, IFN-γ and IL-17. We determined the concentrations of TNF-α, IFN-γ and IL-17 in the supernatant of colonic mucosal homogenates using an enzyme-linked immunosorbent assay (ELISA) kit for TNF-α, IFN-γ and IL-17 (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Subtractive readings at 550 nm from the readings at 450 nm were converted to pg/ml using values obtained from standard curves generated with varying concentrations of recombinant TNF-α, IFN-γ and IL-17.

Immunohistochemical and immunofluorescence staining of MT and F4/80 in colonic mucosa. After 24-h fixation in formalin, samples were embedded in paraffin, after which 4-μm-thick sections were cut using a microtome cryostat and mounted on MAS-coated slides. We performed antigen retrieval using proteinase K solution, and the sections were rinsed with distilled water for 5 min, followed by incubation with 3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. After incubation, the sections were washed in phosphate-buffered saline (PBS)-Tween-20 for 5 min. Non-specific binding was blocked by incubating the slides with Dako Cytomation protein block (Dako, Tokyo, Japan) for 30 min at room temperature. The sections were then incubated with primary antibody against MTs (Dako, Glostrup, Denmark) diluted at 1:50 and F4/80 (Novus Biologicals, Inc., Littleton, CO) diluted at 1:200 with antibody dilution (Dako) overnight at 4˚C. The sections were washed 3 times in PBS-Tween-20 for 5 min and incubated with secondary antibody [Histofine Simple Stain mouse MAX PO (rabbit); Nichirei Biosciences, Inc., Tokyo, Japan] for 30 min at room temperature. Unbound antibodies were removed by 3 washes in PBS for 5 min, and bound antibodies were visualized using diaminobenzidine as a chromogen substrate reagent. Negative controls for non-specific binding incubated with secondary antibodies were confirmed to produce no signal. All sections were counterstained with hematoxylin. Sections were finally dehydrated, cleared and coverslipped.

For the immunofluorescence staining of MTs and F4/80 (diluted at 1:200; Novus Biologicals, Inc.), bound antibodies were visualized using the secondary antibodies, anti-rabbit Alexa 594 (Molecular Probes, Inc., Eugene, OR) and anti-rat Alexa 488 (Molecular Probes), respectively, diluted at 1:1,000. Negative controls used for non-specific binding and incubated with secondary antibodies were confirmed to produce no signal. Fluorescence staining was observed under an inverted fluorescence microscope (IX70-23FL/DIC-SP; Olympus, Tokyo, Japan).

F4/80-positive cells in colonic mucosa. We evaluated the number of F4/80-positive cells in the intestinal mucosa of wild-type and MT-I/II knockout mice following DSS-induced colitis. The number of positively stained cells in the intestinal mucosa was counted in 50 high-power fields per section under a microscope, as described previously (33).

Isolation of resident peritoneal macrophages and determination of cytokine production. Resident peritoneal macrophages were obtained by peritoneal lavage using 10 ml of PBS from both wild-type and MT-I/II knockout mice. Cells were plated at $5x10^5$ cells/well (24-well plate) and incubated for 2 h at 37˚C in RPMI-1640 medium with 5% fetal bovine serum (FBS), 2 mM glutamine, 50 mg/ml streptomycin and 50 U/ml penicillin (all obtained from Life Technologies Corp.). The cells were washed twice with PBS to remove non-adherent cells and cultured in RPMI-1640 for 24 h at 37˚C. Subsequently, the cells were stimulated with LPS (*Escherichia coli* 055:B5; Sigma-Aldrich Corp., St. Louis, MO) at 10 ng/ml. After a 12-h incubation with LPS, cell supernatants were collected and stored at -70˚C. TNF-α, IFN-γ and IL-17 levels in culture supernatants were determined using an ELISA kit according to the manufacturer's instructions (R&D Systems).

Statistical analysis. The results in this study are presented as the means ± standard error of the mean (SEM). Overall differences between the groups were determined by one-way analysis of variance (ANOVA). If the results from one-way ANOVA were significant, the differences between individual groups were analyzed by Bonferroni's multiple comparisons test. P-values <0.05 were considered to indicate statistically significant differences. All analyses were performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA) on a Windows-based computer.

Results

Expression of MT in inflamed colonic mucosa. In order to investigate MT-I/II expression in the inflamed mucosa following the administration of DSS, we examined the expression of colonic MT-I/II mRNA using real-time PCR analysis. The results confirmed that the expression of MT-I/II was significantly higher in the inflamed colonic mucosa in the model of DSS-induced colitis (Fig. 1A).

Body weight changes, DAI scores and colon length following DSS administration. The intake of DSS solution was moni-

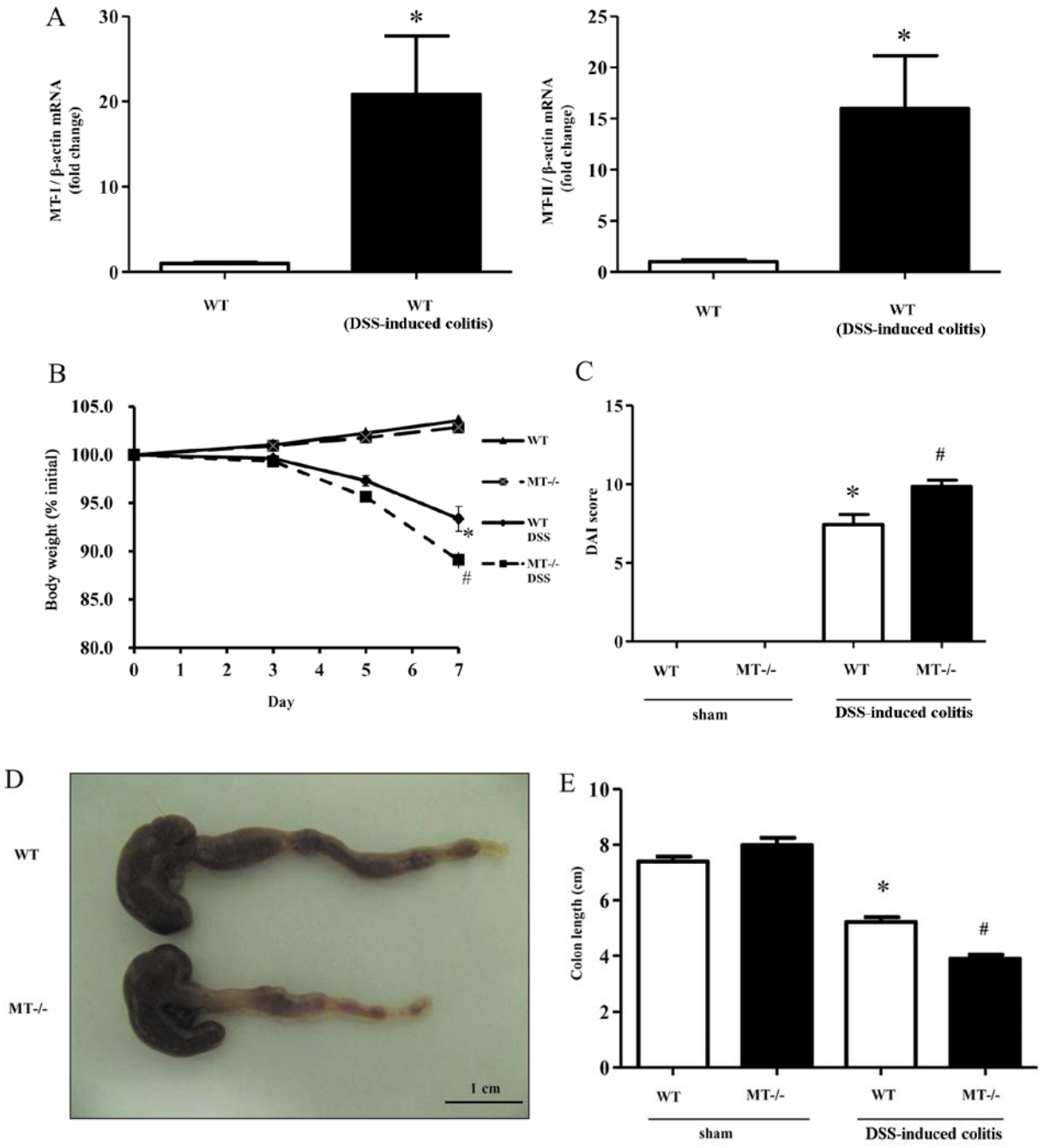

Figure 1. (A) Expression of colonic MT-I/II mRNA in normal mice and in the mouse model of dextran sulfate sodium (DSS)-induced colitis. Real-time PCR analysis confirmed the upregulation of MT-I/II expression following DSS-induced colitis. Both columns represent the means ± standard error of the mean (SEM). n=8. *P<0.05 vs. wild-type mice. (B) Changes in body weight following treatment with DSS. MT-I/II knockout mice showed a marked decrease in body weight. *P<0.05 vs. wild-type mice treated with the vehicle, #P<0.05 vs. wild-type mice treated with DSS. (C) Disease activity index (DAI) of MT-I/II knockout mice was significantly higher than that of wild-type mice. Each column represents the means ± SEM. n=8. *P<0.05 vs. wild-type mice treated with the vehicle; #P<0.05 vs. wild-type mice treated with DSS. (D) Representative macroscopic findings. MT-I/II knockout mice showed marked colon shortening. (E) Colon length following treatment with DSS. Shown is the average colon length. Colon length in MT-I/II knockout mice was significantly shorter than that of wild-type mice. Both columns represent the means ± SEM. n=8. *P<0.05 vs. wild-type mice treated with the vehicle; #P<0.05 vs. wild-type mice treated with DSS. WT, wild-type; $MT^{-/-}$, MT-I/II knockout (MT null) mice.

tored throughout the experiments and was found to be similar among the experimental groups (data not shown). The mice exposed to 2.0% DSS developed symptoms of acute colitis, with diarrhea accompanied by rectal bleeding and body weight loss. Decreases in body weight were significantly greater in the MT-I/II knockout mice in comparison to the wild-type mice (Fig. 1B). DAI scores in the DSS-treated mice, determined by weight loss, stool consistency and blood in stool, were significantly increased in the MT-I/II knockout mice compared to the wild-type mice (Fig. 1C).

Moreover, colon length is known to be reduced in DSS colitis along with the exacerbation of intestinal inflammation. In this study, MT-I/II knockout mice showed marked colon shortening 7 days after DSS administration (Fig. 1D).

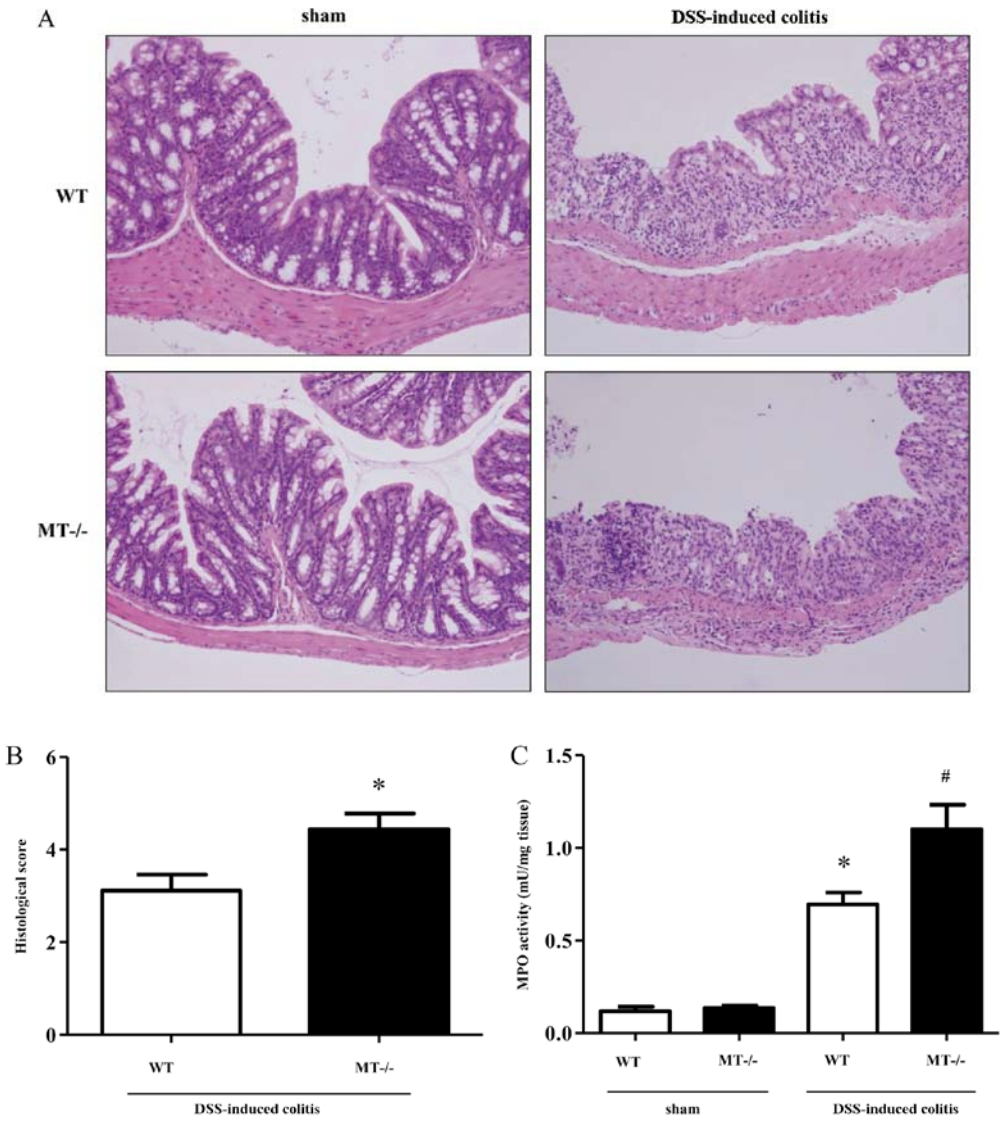

Figure 2. (A) Histological appearance of the colon in dextran sulfate sodium (DSS)-treated MT-I/II knockout mice. Magnification, x100. Hematoxylin and eosin staining. (B) Histological scores of wild-type mice were significantly lower than those of MT-I/II knockout mice. (C) Levels of myeloperoxidase (MPO) activity were elevated in MT-I/II knockout mice, as compared to wild-type mice treated with DSS. Each column represents the means ± SEM. n=8. *P<0.05 vs. wild-type mice treated with the vehicle; #P<0.05 vs. wild-type mice treated with DSS. WT, wild-type; MT-/-, MT-I/II knockout (MT null) mice.

Colon length following DSS administration was significantly decreased in the MT-I/II knockout mice compared to the wild-type mice (Fig. 1E). These data suggest that MTs play a crucial role in the development of intestinal inflammation.

Histology and neutrophil accumulation following DSS administration. The protective effects of MTs were also confirmed by histological analysis. Fig. 2A shows the typical histological appearance of wild-type and MT-I/II knockout mice. In the wild-type mice, the administration of 2% DSS for 7 days resulted in colonic ulceration associated with large areas of epithelial crypt loss and inflammatory cell infiltration throughout the mucosa. On the other hand, MT deficiency resulted in larger erosions with a high degree of inflammatory cell infiltration. The histological scores reflected these findings (Fig. 2B).

Tissue-associated MPO activity (as an index of polymorphonuclear leukocyte accumulation) in the colonic mucosa increased from 0.130±0.079 mU/mg protein (basal concentration) to 0.903±0.114 mU/mg protein in the wild-type mice 7 days following the administration of DSS (Fig. 2B). MPO activity increased in the wild-type mice following DSS administration, and was significantly elevated in the DSS-treated MT-I/II knockout mice (Fig. 2B).

Colonic mRNA expression and mucosal concentrations of various inflammatory cytokines following DSS administration. The expression of inflammatory cytokines in the colonic mucosa following DSS treatment was determined using real-time PCR and ELISA. mRNA expression levels of inflammatory cytokines (TNF-α, IFN-γ and IL-17) were significantly increased in the colonic mucosal tissue of the MT-I/II knockout mice compared with the wild-type mice following the administration of DSS (Fig. 3). In agreement with these results, the colonic mucosal concentrations of these inflammatory cytokines (TNF-α, IFN-γ and IL-17) in the DSS-treated MT-I/II knockout mice were significantly higher, as compared with the DSS-treated wild-type mice (Fig. 4).

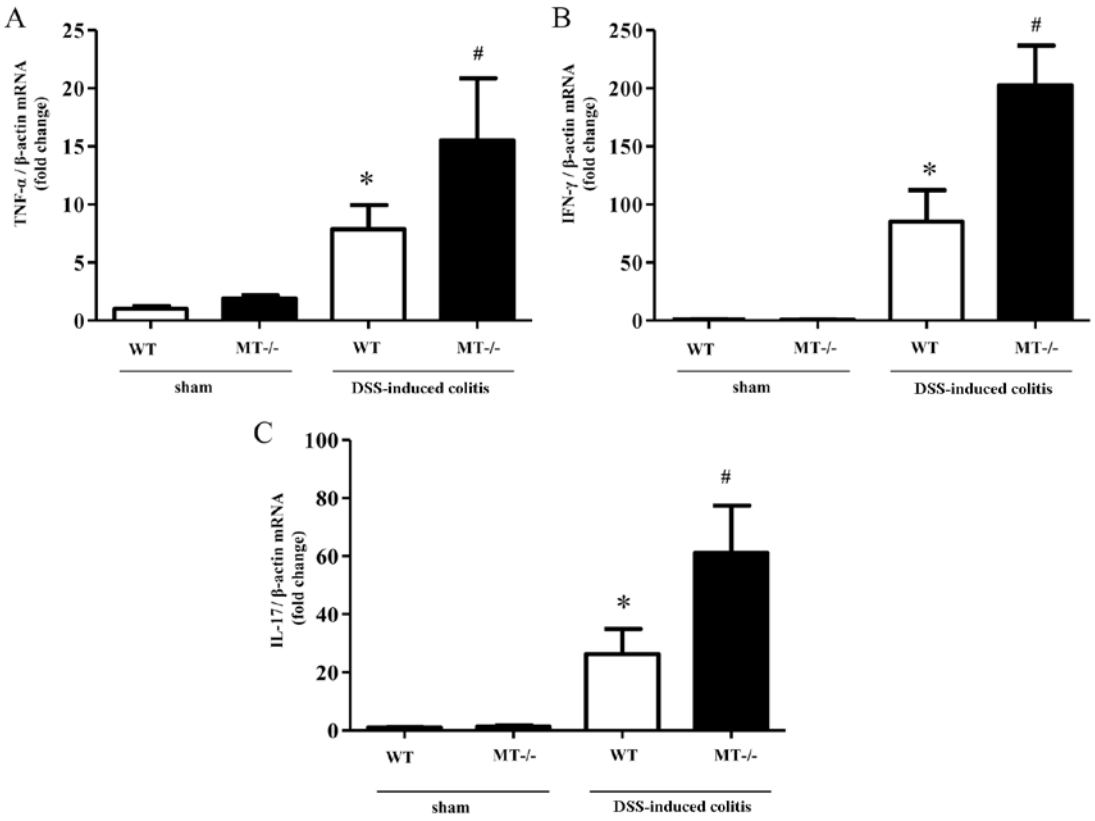

Figure 3. mRNA cytokine expression in colonic mucosal tissue. Inflammatory cytokine [(A) TNF-α, (B) IFN-γ and (C) IL-17] levels were significantly higher in colonic mucosal tissue of MT-I/II knockout mice. Each column represents the means ± SEM. n=8. *P<0.05 vs. wild-type mice treated with the vehicle; #P<0.05 vs. wild-type mice treated with dextran sulfate sodium (DSS). WT, wild-type; MT-/-, MT-I/II knockout (MT null) mice.

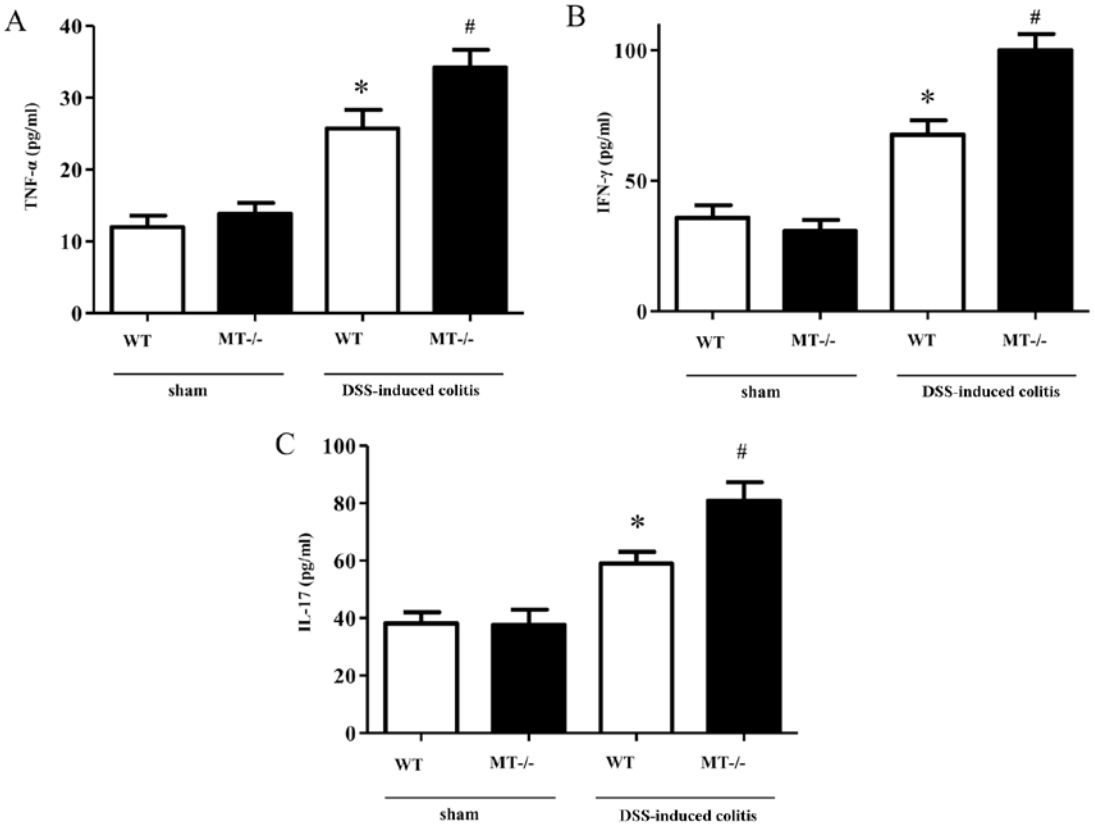

Figure 4. Production of cytokines in colonic mucosal tissue as shown by ELISA. Levels of inflammatory cytokines [(A) TNF-α, (B) IFN-γ and (C) IL-17] were significantly elevated in colonic mucosal tissue of MT-I/II knockout mice. Each column represents the means ± SEM. n=8. *P<0.05 vs. wild-type mice treated with the vehicle; #P<0.05 vs. wild-type mice treated with dextran sulfate sodium (DSS). WT, wild-type; MT-/-, MT-I/II knockout (MT null) mice.

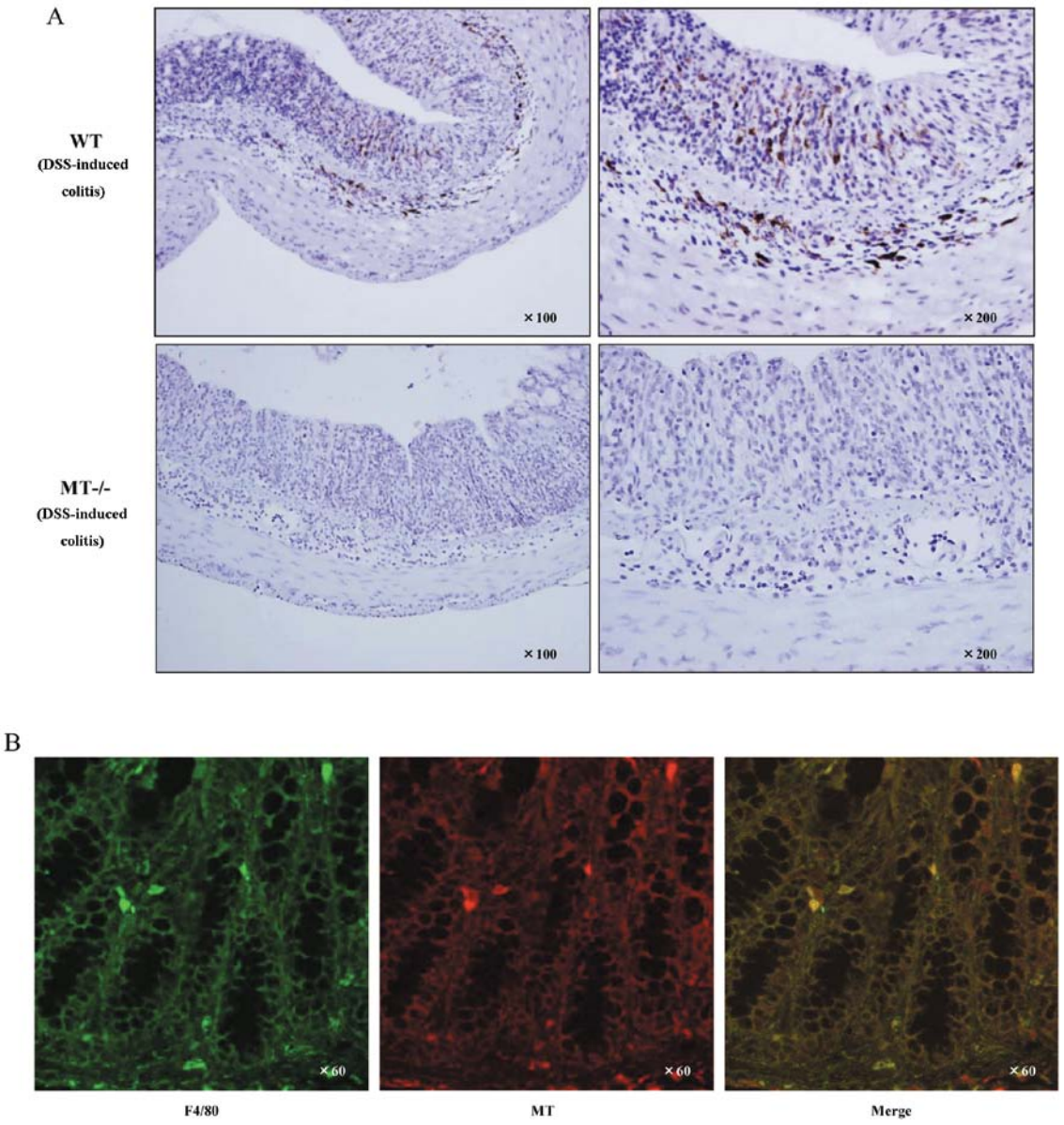

Figure 5. (A) Immunohistochemical staining of MTs. MT-positive cells were mainly localized in the lamina propria and submucosal layer as shown by immunohistochemical examination of wild-type mice, but MTs were not expressed in the MT-I/II knockout mice. (B) Localization of colonic MT expression was analyzed using immunofluorescence staining. MT and F4/80 were visualized under a laser scanning confocal microscope using the secondary antibodies, anti-rabbit Alexa 594 and anti-rat Alexa 488. A representative image of 3 separate experiments is shown. MT-positive cells mostly corresponded with F4/80-positive macrophages. WT, wild-type; MT-/-, MT-I/II knockout (MT null) mice.

Immunohistochemical and immunofluorescence staining of MTs in inflamed colonic mucosa. MTs are mainly localized in mononuclear cells in the lamina propria and submucosal layer, and these MT-positive cells were elevated in the DSS-treated wild-type mice. By contrast, MT-positive cells were not detected in the colons of MT-I/II knockout mice before and after DSS administration (Fig. 5A).

In addition, in order to investigate the detailed localization of MT expression in the colonic mucosa, we conducted immunofluorescence staining of the colonic mucosa. MT-positive cells largely corresponded with F4/80-positive macrophages (Fig. 5B).

Number of F4/80-positive cells in inflamed colonic mucosa. F4/80-positive cells were mainly localized in the lamina propria and submucosal layer in wild-type and MT-I/II knockout mice after DSS administration (Fig. 6A). Furthermore, we evaluated the number of the F4/80-positive cells in the intestinal mucosa of wild-type mice and MT-I/II knockout mice. No significant differences were observed in the number of F4/80-positive cells between the DSS-treated wild-type and MT-I/II knockout mice (Fig. 6B).

Production of inflammatory cytokines in peritoneal macrophages following LPS treatment. In order to determine the production of inflammatory cytokines (TNF-α, IFN-γ and IL-17) in murine peritoneal macrophages following treatment with LPS, we measured the levels of these cytokines in the supernatant of cultured cells using ELISA. The concentrations of the inflammatory cytokines (TNF-α, IFN-γ and IL-17)

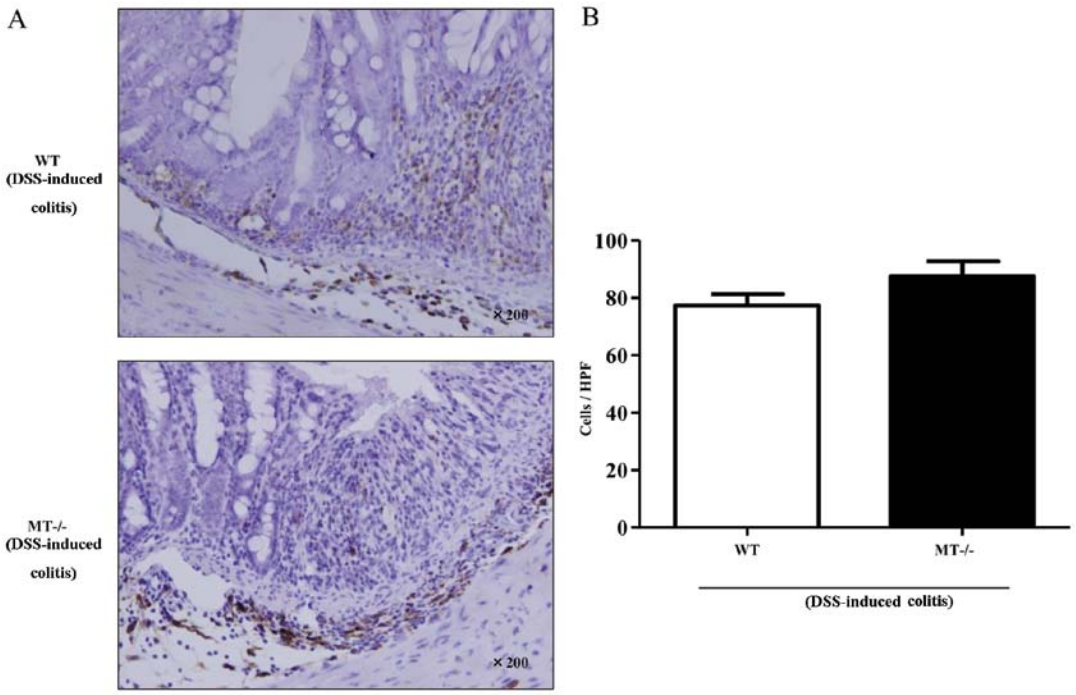

Figure 6. (A) Immunohistochemical staining of F4/80-positive macrophages. F4/80-positive cells were mainly localized in the lamina propria and submucosal layer as shown by immunohistochemical examination of wild-type and MT-I/II knockout mice. (B) The number of macrophages in wild-type and MT-I/II knockout mice was approximately equal. Cells/HPF, number of cells per high power field; WT, wild-type; MT-/-, MT-I/II knockout (MT null) mice.

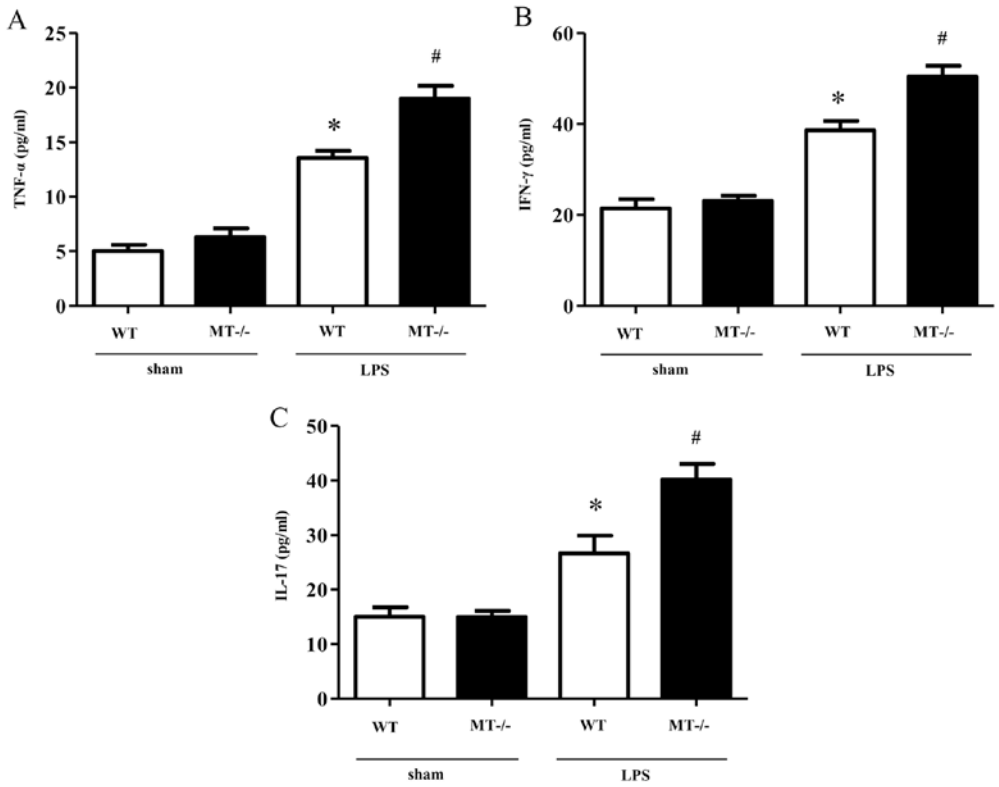

Figure 7. Production of cytokines in the supernatant of isolated peritoneal macrophages. Levels of inflammatory cytokines [(A) TNF-α, (B) IFN-γ and (C) IL-17] were significantly higher in the supernatants of macrophages derived from MT-I/II knockout mice when compared to those of wild-type mice. Each column represents the means ± SEM. n=8. *P<0.05 vs. wild-type mice treated with vehicle; $^{\#}$P<0.05 vs. wild-type mice treated with lipopolysaccharide (LPS). WT, wild-type; MT-/-, MT-I/II knockout (MT null) mice.

in the LPS-treated macrophages isolated from the MT-I/II knockout mice were significantly higher as compared with the LPS-treated macrophages derived from the wild-type mice (Fig. 7).

Discussion

In the current study, intestinal injury was assessed based on a variety of factors, including body weight, disease activity score, colon length and histology. In each assessment, colonic injury was found to be significantly aggravated in the DSS-treated MT-I/II knockout mice, as compared with the DSS-treated wild-type mice. Histological analysis also showed an enhanced infiltration of inflammatory cells, particularly neutrophils and mononuclear cells, as well as mucosal epithelial cell disruption in the DSS-treated MT-I/II knockout mice. Enhanced intestinal injury and neutrophil accumulation in MT-I/II knockout mice were also confirmed by the measurement of histological scores using a previously reported grading system (30,31) and MPO activity of the colon, respectively.

A number of previous studies have described the role of MTs in the inflammatory response to DSS. Tran *et al* (20) first demonstrated that MT-deficient mice had significantly lower DAI when compared with wild-type mice in a model of DSS-induced colitis. However, the results from our study are not in agreement with the histological assessment made in the study by Tran *et al*. Another study by Oz *et al* (19) reported no differences in histological damage following treatment with DSS among wild-type mice, MT-deficient mice and MT transgenic mice. However, their study did not include the assessment of DAI. On the other hand, our data suggest that MT deficiency results in disease exacerbation in a mouse model of DSS-induced colitis, and that MTs play a protective role in the development of intestinal inflammation. While considering these results, we noted differences in the manufacture of DSS and *vivarium* in these studies, and it has been reported that these factors can lead to differences in the severity of DSS-induced colitis (34,35).

As regards MT expression in intestinal inflammation, there are conflicting reports on the role of MTs in intestinal inflammation. Mulder *et al* (36) demonstrated that MT expression was decreased in the colonic mucosa of patients with IBD. Kruidenier *et al* (37) also reported that patients with IBD had decreased MT expression levels in the colon. By contrast, Lih-Brody *et al* (38) and Bruwer *et al* (39) reported that MT expression was markedly elevated in the colonic mucosa of patients with IBD. Similarly, MT-I/II expression was significantly higher in the inflamed colonic mucosa in mice with DSS-induced colitis, as compared with normal colonic mucosa in wild-type mice in the present study.

In the present study, in order to investigate the localization of MTs in the intestine, immunohistochemical staining was performed with murine colonic samples using anti-MT-I/II antibody. MT-I/II expression was mainly observed in mononuclear cells in the lamina propria and submucosal layer of the murine intestine. Further examination using immunofluorescence staining revealed that these mononuclear cells were F4/80-positive macrophages. Although the localization of MTs in the gastrointestinal tract is not yet fully understood, Kruidenier *et al* (37) demonstrated that MTs are mainly expressed in the small intestinal epithelium, not in cells in the lamina propria. By contrast, a previous study by Al-Gindan *et al* (40) demonstrated that MT expression is localized in submucosal cells in the colonic mucosa in rats, similar to our results.

As regards the expression of MTs in macrophages, there is an extensive amount of evidence suggesting that alveolar macrophages show strong MT expression. Mehta *et al* (41) demonstrated that the reduction in MT expression induced by zinc deficiency in alveolar macrophages inhibits phagocytic function, indicating that MTs mediate innate immune function. Pankhurst *et al* (42) also reported that MT-expressing macrophages in the murine brain may play a crucial role in the regulation of Th1/Th2 cytokine balance. In this study, the production of Th1, Th2 and Th17 cytokines was significantly elevated in the MT-I/II knockout mice, indicating that MTs possibly regulate the production of these cytokines. It is known that TNF-α, IFN-γ and IL-17 are mainly released by helper T cells (Th-1, Th-2 and Th-17) and macrophages (43,44), and large amounts of cytokine expression lead to severe mucosal inflammation in ulcerative colitis and Crohn's disease (45). In the present study, we evaluated the function of macrophages derived from MT-deficient mice, as the number of infiltrating F4/80-positive macrophages in the intestinal mucosa of mice treated with DSS did not differ between the wild-type and MT-I/II knockout mice. We found that the production of inflammatory cytokines (TNF-α, IFN-γ and IL-17) in the supernatant of macrophages isolated from MT-I/II knockout mice following LPS stimulation was significantly higher compared to wild-type mice. Thus, MTs appear to play an important role in the anti-inflammatory function of macrophages and MT-expressing macrophages may play a protective role in the pathogenesis of IBDs.

In conclusion, our results suggest that MTs play a protective role against colonic mucosal inflammation in a mouse model of DSS-induced colitis through their anti-inflammatory function in macrophages, indicating that endogenous MTs play an important role in the protection of the intestinal mucosa. In the future, MTs may prove to be novel therapeutic target molecules in IBDs.

Acknowledgements

The authors thank Dr Chiharu Tohyama (The University of Tokyo) for providing the MT-I/II knockout mice.

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