Table I. *Expression of surface Ags on BM-derived different subsets of macrophage*[a]

	ΔMFI			
	WT		KO	
	GM-Mϕ	M-Mϕ	GM-Mϕ	M-Mϕ
CD11b	1174.2 ± 155.5*	509.5 ± 58.7	1229.7 ± 15.5*	388.2 ± 58.5
Gr-1	93.1 ± 3.4*	0.0 ± 0.0	63.7 ± 7.6*	0.0 ± 0.0
CD80	290.4 ± 34.2*	73.4 ± 27.5	383.0 ± 4.1*	38.6 ± 2.5
CD86	14.5 ± 1.6	36.2 ± 8.1	26.7 ± 0.9	35.9 ± 3.4
TLR2	348.6 ± 18.6	342.4 ± 118.8	386.0 ± 48.8	220.0 ± 4.4
TLR4/MD2	7.6 ± 2.1	18.4 ± 0.8	8.7 ± 4.3	18.8 ± 4.8
MHC-II	20.6 ± 3.7*	1.7 ± 0.4	24.4 ± 5.9*	1.5 ± 0.3

[a] Data indicated as ΔMFI (geometric mean fluorescence intensity (MFI) of each Ab staining minus MFI of control IgG staining), and are expressed as mean ± SEM of five independent experiments. *, $p < 0.01$ compared with M-Mϕ (Sheffe's test)

were almost undetectable in GM-Mϕ and M-Mϕ from both WT and IL-$10^{-/-}$ mice (data not shown). Upon stimulation with *E. coli*, their expressions were detected in GM-Mϕ from both WT and IL-$10^{-/-}$ mice, and expression levels in GM-Mϕ from IL-$10^{-/-}$ mice were ~3- to 4-fold higher than levels in WT mice (Fig. 3*C*). In contrast, expression levels of those genes were quite different in M-Mϕ from WT and IL-$10^{-/-}$ mice. These transcripts remained at very low levels in WT M-Mϕ even after stimulation; however, IL-$10^{-/-}$ M-Mϕ showed markedly high expressions of IL-12 and IL-23 genes (Fig. 3*C*).

These results suggest that GM-Mϕ can produce IL-12 and IL-23 in response to bacterial stimuli, whereas M-Mϕ cannot produce these cytokines but rather produce anti-inflammatory cytokine IL-10. The results also suggested that endogenous IL-10 strongly contributes to inhibition of IL-12p70 production in M-Mϕ but not GM-Mϕ. Thus, IL-$10^{-/-}$ M-Mϕ may contribute to Th1 polarization by producing IL-12p70 upon stimulation with bacteria.

Exogenous IL-10 supplementation at the time of stimulation with whole bacteria Ag inhibits abnormal IL-12p70 hyperproduction by IL-$10^{-/-}$ M-Mϕ

Because M-Mϕ, but not GM-Mϕ, from IL-$10^{-/-}$ mice showed abnormal IL-12 and IL-23 hyperproduction in response to stimulation with *E. coli*, we further examined how absence of IL-10 led to IL-12 and IL-23 hyperproduction from M-Mϕ. M-Mϕ from IL-$10^{-/-}$ mice were stimulated with heat-killed bacterial Ags concomitant with a supplementation of exogenous IL-10 (Fig. 4*A*). Abnormal IL-12p70 hyperproduction by M-Mϕ from IL-$10^{-/-}$ mice was completely suppressed by exogenous IL-10 in a dose-dependent manner (Fig. 4*B*). In addition, IL-10 had similar inhibitory effects on productions of other proinflammatory cytokines (TNF-α and IL-6) from IL-$10^{-/-}$ Mϕ. These findings were consistent with a previous report showing that IL-10 inhibited production of several proinflammatory cytokines by Mϕ, including IL-12 (28). These results suggest that IL-10 inhibits the production of proinflammatory cytokines by M-Mϕ in response to stimulation with whole bacteria Ags.

Exogenous IL-10 supplementation during the differentiation process attenuates abnormal IL-12p70 hyperproduction by IL-$10^{-/-}$ M-Mϕ

As described, IL-10 production by M-Mϕ in response to bacteria is important for suppression of IL-12p70 production, as well as for other cytokine productions; however, how IL-10 acts on the differentiation process of BM CD11b$^+$ cells still remains unclear. Therefore, we examined the role of IL-10 in differentiation of M-Mϕ from BM CD11b$^+$ cells. BM CD11b$^+$ cells from IL-$10^{-/-}$ mice were differentiated into M-Mϕ with M-CSF in the presence of exogenous IL-10. Polarized M-Mϕ were thoroughly washed to remove any residual IL-10, and then stimulated by heat-killed *E. coli* without exogenous IL-10 (Fig. 5*A*). Interestingly, M-Mϕ differentiated from IL-$10^{-/-}$ mice in the presence of M-CSF and exogenous IL-10 was unable to induce large amounts of IL-12p70 production in response to stimulation with *E. coli*, although exogenous IL-10 was removed from the culture supernatant before bacterial Ags were added (Fig. 5*B*).

Contrary to the production of IL-12p70, the other proinflammatory cytokines (TNF-α and IL-6) were only partially or not significantly suppressed by IL-10 supplementation during the differentiation process. Moreover, consistent with IL-12p70 results, levels of IL-12p35, IL-12p40, and IL-23p19 mRNA transcripts were significantly reduced in IL-$10^{-/-}$ M-Mϕ differentiated in the presence of exogenous IL-10 (Fig. 5*C*). These results suggest that endogenous IL-10 is an essential cytokine for functional differentiation of M-Mϕ, especially for maturation of the phenotype as anti-inflammatory Mϕ, which cannot produce IL-12p70 while producing large amounts of IL-10.

CLPMϕ but not splenic Mϕ show functional similarity to BM-derived M-Mϕ in the production of IL-10 and IL-12

It became evident that in vitro differentiated M-Mϕ, but not GM-Mϕ, from IL-$10^{-/-}$ mice showed abnormal responses to whole bacteria Ags. Hence, we further analyzed CLPMϕ from WT and IL-$10^{-/-}$ mice to investigate how intestinal Mϕ act in vivo and contribute to trigger and develop Th1-dominant inflammation in IL-$10^{-/-}$ mice. CLPMϕ from WT mice did not produce IL-12p70 upon stimulation with heat-killed *E. coli*, but instead produced large amounts of IL-10, and the levels were similar to those of BM-derived M-Mϕ (Fig. 6*A*). In contrast, CLPMϕ from IL-$10^{-/-}$ mice produced significantly larger amounts of IL-12p70 in response to stimulation with the bacteria, and levels were similar to those of BM-derived M-Mϕ from IL-$10^{-/-}$ mice (Fig. 6*A*). In contrast to CLPMϕ, abnormal IL-12p70 hyperproductions by bacteria were not observed in splenic Mϕ from IL-$10^{-/-}$ mice, although TNF-α induction levels were similar to those of CLPMϕ (Fig. 6*A*). Similar results were obtained when CLPMϕ from WT and IL-$10^{-/-}$ mice were stimulated with heat-killed *E. faecalis* (data not shown).

These results suggest that CLPMϕ revealed a functional similarity to that of M-Mϕ, that CLPMϕ from WT mice act as anti-inflammatory Mϕ via production of large amounts of IL-10, and that CLPMϕ from IL-$10^{-/-}$ mice contribute to the development of Th1-dominant colitis via the abnormal hyperproduction of IL-12p70.

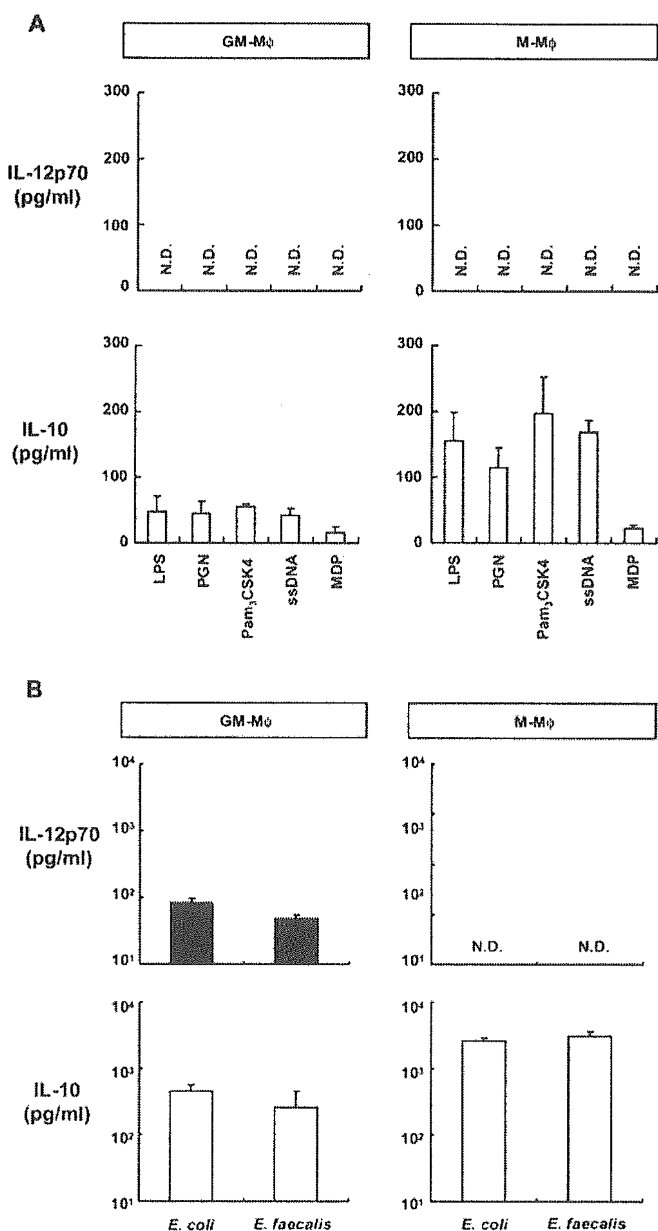

FIGURE 2. BM-derived M-Mϕ from WT mice reveal an anti-inflammatory phenotype in response to PAMPs and whole bacterial Ags. *A*, Polarized GM-Mϕ and M-Mϕ (1×10^5 cells) from WT mice were stimulated with LPS (100 ng/ml), PGN (2 μg/ml), Pam_3CSK_4 (500 ng/ml), *E. coli* ssDNA (10 μg/ml), or MDP (10 μg/ml) for 24 h. The amounts of IL-12p70 and IL-10 in the culture supernatants were measured using a CBA kit. Data are expressed as the mean ± SEM from five independent experiments for LPS, PGN, Pam_3CSK_4, and ssDNA and three independent experiments for MDP. *B*, GM-Mϕ and M-Mϕ (1×10^5 cells) from WT mice were stimulated with a heat-killed Gram-negative strain of *E. coli* or a heat-killed Gram-positive strain of *E. faecalis* (MOI = 100). Data are expressed as the mean ± SEM from seven independent experiments. N.D., Not detected.

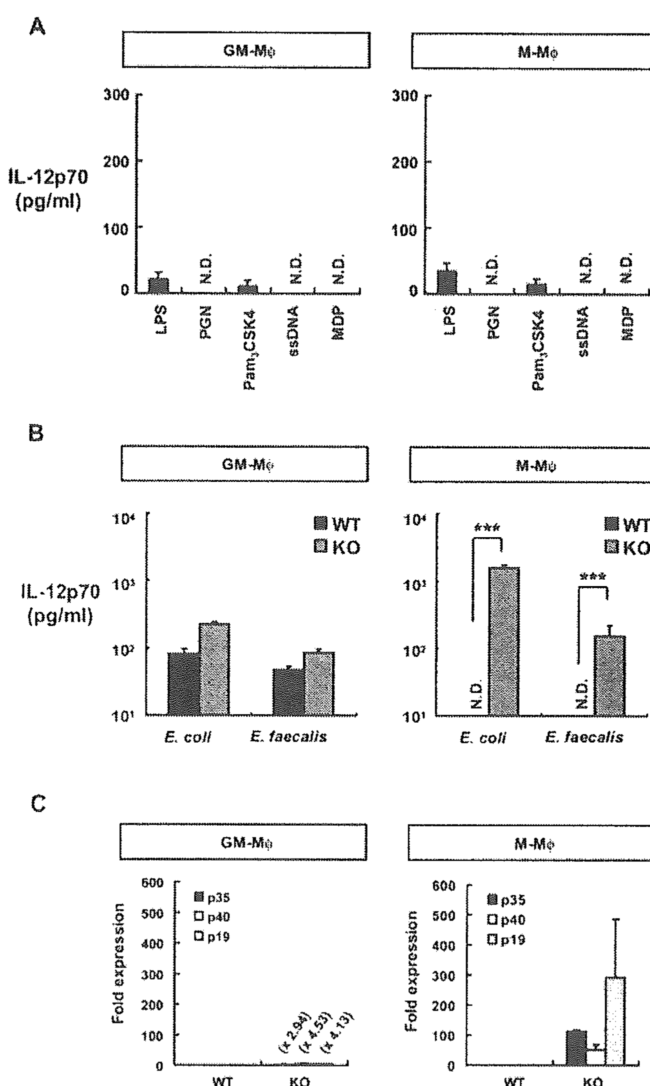

FIGURE 3. BM-derived M-Mϕ from IL-$10^{-/-}$ mice produced large amounts of IL-12p70 in response to whole bacteria Ags. *A*, GM-CSF- or M-CSF-induced Mϕ (1×10^5 cells) from IL-$10^{-/-}$ mice (KO) were stimulated with LPS (100 ng/ml), PGN (2 μg/ml), Pam_3CSK_4 (500 ng/ml), *E. coli* ssDNA (10 μg/ml), or MDP (10 μg/ml) for 24 h. Data are expressed as the mean ± SEM from five independent experiments for LPS, PGN, Pam_3CSK_4, ssDNA and three independent experiments for MDP. *B*, GM-CSF- or M-CSF-induced Mϕ (1×10^5 cells) from WT and mice were stimulated with heat-killed *E. coli* or *E. faecalis* (MOI = 100) for 24 h. Data are expressed as the mean ± SEM from seven independent experiments. N.D., Not detected. ***, $p < 0.001$ compared with WT Mϕ (Student's *t* test). *C*, GM-Mϕ and M-Mϕ (1×10^5 cells) were stimulated by heat-killed *E. coli* (MOI = 100) for 8 h. The gene expressions of IL-12p35, IL-12p40, and IL-23p19 were analyzed by real-time quantitative PCR and normalized by the amount of β-actin transcripts. Data indicate the fold expression compared with WT mice and are expressed as the mean ± SEM from three independent experiments.

Therefore, we next assessed the expression of M-CSF and GM-CSF in colonic tissues and spleen because M-CSF and GM-CSF are different in their activity to induce anti-inflammatory Mϕ as described. M-CSF to GM-CSF expression level ratios in murine colonic tissues were dramatically higher than in spleen (Fig. 6*B*).

These results demonstrate that M-CSF rich environment in colonic tissues may contribute the differentiation of intestinal Mϕ into anti-inflammatory M-Mϕ phenotype.

Discussion

The results we present revealed that CLPMϕ and M-CSF-induced M-Mϕ in WT mice produce large amounts of IL-10, but not IL-12 and IL-23 upon stimulation with whole bacteria Ags. In contrast, GM-CSF-induced GM-Mϕ in WT mice produce IL-12 and IL-23 despite of IL-10 production. In contrast, we first demonstrated that CLPMϕ and BM-derived M-Mϕ in IL-$10^{-/-}$ mice produced abnormal large amounts of IL-12 and IL-23 upon stimulation with bacteria, but splenic Mϕ and BM-derived GM-Mϕ in IL-$10^{-/-}$ mice were not significantly different from those in WT mice. These

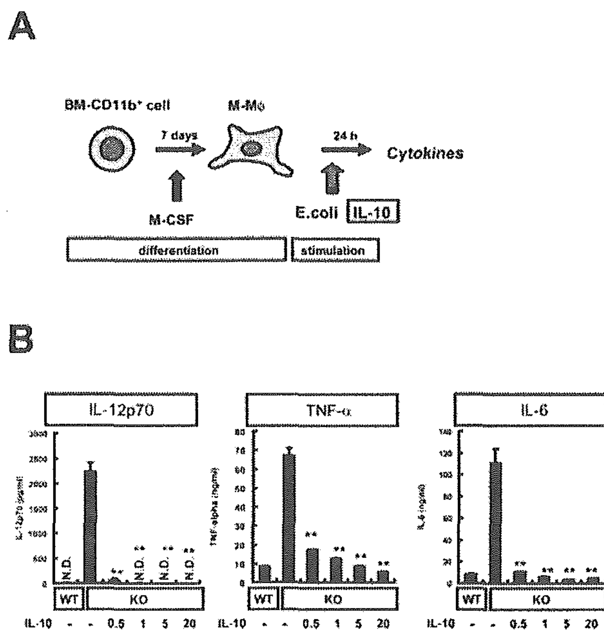

FIGURE 4. Exogenous IL-10 prevents the production of proinflammatory cytokines by M-Mϕ from IL-$10^{-/-}$ mice. *A*, Schema of the experiment. *B*, M-Mϕ (1×10^5 cells) from WT and IL-$10^{-/-}$ mice (KO) were stimulated with heat-killed *E. coli* (MOI = 100) for 24 h with or without various concentrations of exogenous IL-10. The amounts of IL-12p70, TNF-α, and IL-6 in the culture supernatants were measured using a CBA kit. Data are expressed as the mean ± SEM from three independent experiments. N.D., Not detected. **, $p < 0.01$ compared with IL-$10^{-/-}$ Mϕ without IL-10 supplementation (Dunnett's test).

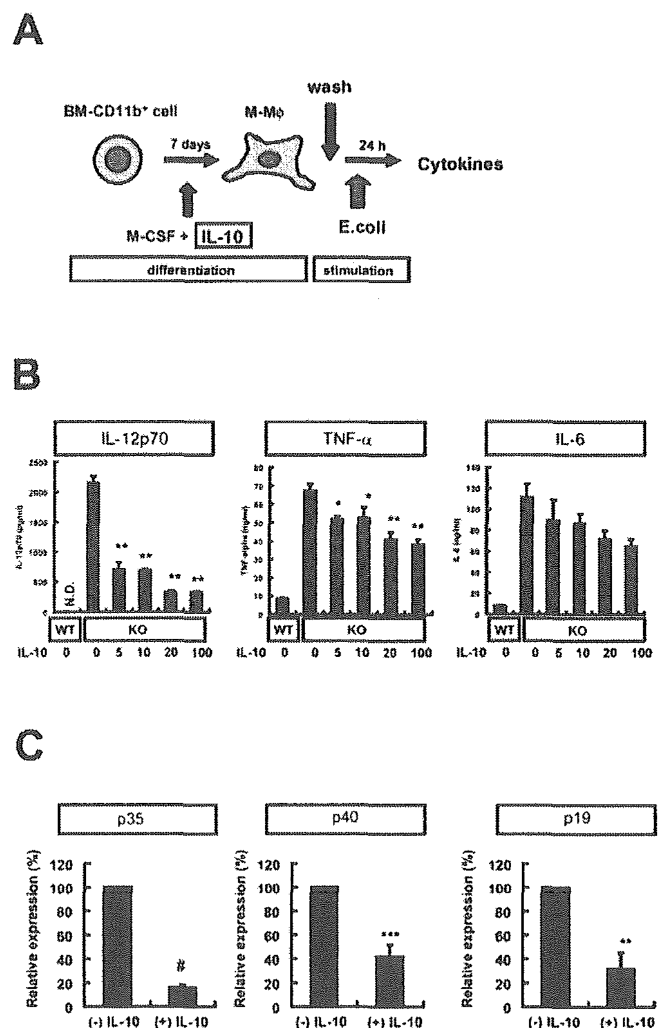

FIGURE 5. IL-10 supplementation during the differentiation process of M-Mϕ improves the abnormal IL-12 production upon stimulation with whole bacterial Ags. *A*, Schema of the experiment. *B*, BM CD11b$^+$ cells from IL-$10^{-/-}$ mice (KO) were differentiated with 20 ng/ml M-CSF and various concentrations of exogenous IL-10 (0–100 ng/ml) for 7 days. The M-Mϕ were harvested, seeded at 1×10^5 cells/well, and incubated for 15–16 h. The cells were washed three times to remove any residual cytokines and then stimulated by heat-killed *E. coli* (MOI = 100) for 24 h. The amounts of IL-12p70, TNF-α, and IL-6 in the culture supernatants were measured using a CBA kit. Data are expressed as the mean ± SEM from 10 independent experiments. N.D., Not detected. *, $p < 0.05$; **, $p < 0.01$ compared with IL-$10^{-/-}$ Mϕ differentiated without IL-10 supplementation (Dunnett's test). *C*, BM CD11b$^+$ cells from IL-$10^{-/-}$ (KO) were polarized with M-CSF (20 ng/ml) alone or M-CSF plus IL-10 (20 ng/ml). The polarized Mϕ were stimulated by heat-killed *E. coli* (MOI = 100) for 8 h. The mRNA expressions are shown as relative percentages of the levels in knockout mice without IL-10 supplementation. Data are expressed as the mean ± SEM from five independent experiments. **, $p < 0.01$; ***, $p < 0.001$; #, $p < 0.0001$ compared with levels in KO mice without IL-10 supplementation (Student's *t* test).

results indicate that CLPMϕ usually acts as anti-inflammatory Mϕ, however, CLPMϕ in IL-$10^{-/-}$ mice play key roles in Th1-dominant chronic colitis through excess production of IL-12 and IL-23.

In the present study, we demonstrated that GM-Mϕ and M-Mϕ were different not only in morphology or cell surface Ag expression but also in the production of proinflammatory cytokines IL-12, IL-23, and anti-inflammatory cytokine IL-10 in response to heat-killed bacteria, such as *E. coli* and *E. faecalis*. Such differences in cytokine production by Mϕ generated under the influence of M-CSF and GM-CSF were also reported in human monocyte-derived Mϕ. Human monocyte-derived GM-Mϕ show potent Ag-presenting functions, produce IL-12p40 and IL-23p19, but none to low levels of IL-10 in response to mycobacteria and their components, and promote development of Th1 immunity (27, 29, 30). In contrast, human monocyte-derived M-Mϕ show low Ag-presenting activity and produce large amounts of IL-10 but no IL-12 or IL-23 (27, 29, 30). Thus, it is considered that these two subsets of Mϕ play opposite roles both in mice and humans; GM-Mϕ act as proinflammatory and M-Mϕ act as anti-inflammatory Mϕ in response to bacteria. In contrast to mice BM-derived GM-Mϕ, human monocyte-derived GM-Mϕ can produce IL-23 but not IL-12 (29). Reasons for differences between our mice study and previously reported human studies might be attributed to differences in the type of cells used (mouse BM-derived Mϕ and human monocyte-derived Mϕ) or in the stimulus used (*E. coli* and *E. faecalis* vs mycobacteria and their components).

Because the intestinal mucosa of the gut is always exposed to numerous enteric bacteria including both pathogenic and nonpathogenic bacteria, it is considered that the gut may possess regulatory mechanisms preventing excessive inflammatory responses. In fact, it was previously reported that human intestinal Mϕ does not express innate response receptors (31, 32). Although these cells retained their phagocytic and bacteriocidal functions, they did not produce proinflammatory cytokines in response to several inflammatory stimuli such as microbial components (31, 32). Thus, recent studies have suggested that Mϕ located in the intestinal mucosa play important roles in the maintenance of intestinal homeostasis by protecting the host from foreign pathogens (33) and regulating excess immune responses to enteric bacteria (32).

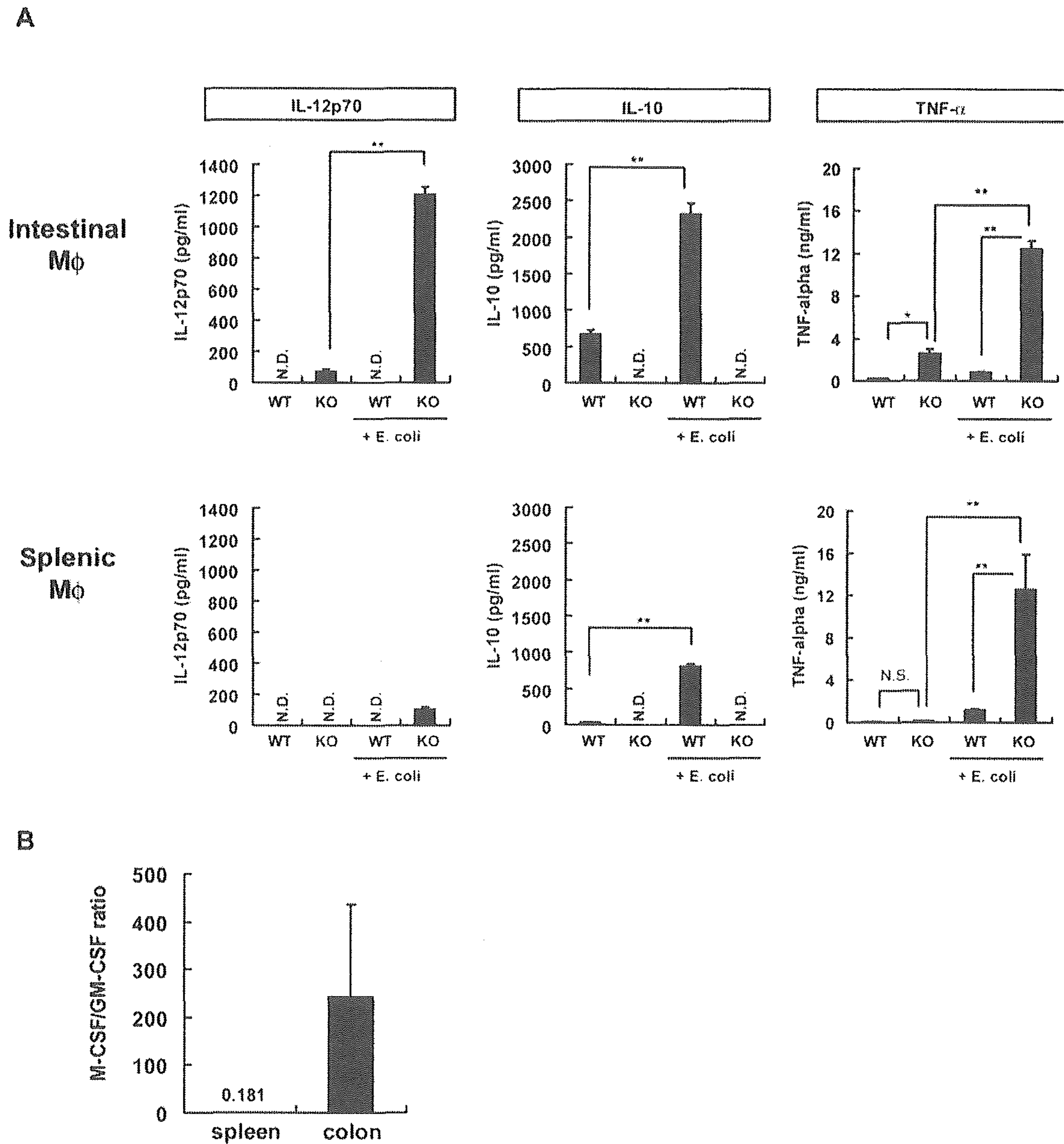

FIGURE 6. CLPMϕ, but not splenic Mϕ, from IL-$10^{-/-}$ mice produce a large amount of IL-12 upon stimulation with whole bacterial Ags. *A*, CLPMϕ and splenic Mϕ were isolated from WT and IL-$10^{-/-}$ mice (KO). Mϕ were seeded at 1×10^5 cells/well and incubated with or without heat-killed *E. coli* (MOI = 100) for 24 h. The amounts of IL-12p70, IL-10, and TNF-α in the culture supernatants were measured using a CBA kit. Data are expressed as the mean ± SEM from six independent experiments. N.D., Not detected; N.S., not significant. $**$, $p < 0.01$ (Sheffe's test). *B*, Expression of M-CSF and GM-CSF in murine tissues from WT mice. Data are expressed as the ratio of M-CSF to GM-CSF expression.

Consistent with these human studies, our present study shows that CLPMϕ in WT mice does not produce proinflammatory cytokines IL-12 and IL-23, and produced just few amounts of TNF-α and IL-6 (data not shown) but large amounts of IL-10 upon stimulation with heat-killed *E. coli* and *E. faecalis* Ags. Thus, CLPMϕ may act as anti-inflammatory Mϕ in vivo, when encountering bacteria. These behaviors of CLPMϕ were very similar to those of in vitro differentiated M-Mϕ. In agreement with this observation, our present study showed that M-CSF to GM-CSF expression level ratios in murine colonic tissues were higher than in other organs, such as spleen. These results suggest that M-CSF-rich conditions in colonic tissues might play an important role in differentiation of CLPMϕ as anti-inflammatory Mϕ. In accordance with this idea, recent studies suggested that M-CSF is an essential growth factor for development of intestinal Mϕ. The number of intestinal Mϕ in M-CSF-deficient *op/op* mice was significantly decreased (33, 34), and M-CSF was expressed in the lamina propria in the human intestine (35).

IL-$10^{-/-}$ mice develop Th1 polarized spontaneous chronic colitis and are widely used as a colitis animal model for human IBDs (14). It has been reported that enteric bacteria play essential roles in onset and development of colitis in IL-$10^{-/-}$ mice, similar to human IBDs (15). However, functional roles of enteric bacteria in development of colitis in IL-$10^{-/-}$ mice have not been identified. We demonstrated in this study that bacteria induce abnormal production of proinflammatory cytokines IL-12 and IL-23 from intestinal Mϕ, but not splenic Mϕ in IL-$10^{-/-}$ mice. Because IL-12 and IL-23 are key cytokines, which induce Th1 immune responses, and IL-12 plays a critical role for the development of colitis in IL-$10^{-/-}$ mice (22, 23), these abnormal responses of intestinal Mϕ in IL-$10^{-/-}$ mice to bacteria may cause Th1 polarization and development of colitis.

In the present study, only stimuli from whole bacteria, but not from PAMPs could induce the production of IL-12p70, IL-12 bioactive form consisted of p35-p40 heterodimer, in differentiated Mϕ from BM CD11b$^+$ cells. In general, TLR ligands such as LPS only induce IL-12p40 subunits, but fail to induce IL-12p70 production from Mϕ without IFN-γ costimulation (36, 37). Consistent with this, we also demonstrated that GM-Mϕ and M-Mϕ produced none or just low levels of IL-12p70 in response to stimulation with various TLR ligands. In contrast, whole bacterial Ags induced IL-12p70 production by GM-Mϕ in WT and IL-10$^{-/-}$ mice, and by M-Mϕ in IL-10$^{-/-}$ mice without IFN-γ. Moreover, intestinal Mϕ from IL-10$^{-/-}$ mice also produced high levels of IL-12p70 in response to stimulation with whole bacteria Ags without IFN-γ, but did not induce IL-12p70 in response to LPS alone (data not shown). These findings imply that TLR ligands and whole bacteria may activate IL-12p70 production through distinct mechanisms. Because whole bacteria are internalized into Mϕ by phagocytosis, it is possible that cell surface receptors involved in phagocytosis are different from TLRs, and can stimulate signaling for IL-12p70 production, and/or internalized bacteria stimulate IL-12p70 production via an intracellular recognition pathway. These are important observations that will help in understanding the pathogenesis of enteric bacteria in development of colitis in IL-10$^{-/-}$ mice, and clarification of these points are underway.

Mϕ and DCs can produce both IL-12 and IL-10, but IL-10 is known to inhibit the production of not only IL-12 but also other proinflammatory cytokines through several transcriptional regulations (38–40). In fact, in the present study, we demonstrated that abnormal IL-12p70 hyperproduction by M-Mϕ in IL-10$^{-/-}$ mice were completely suppressed by exogenous IL-10 supplementation concomitant with bacterial stimulation. These results indicated that the lack of IL-10 production by bacterial stimulation may account for the abnormal IL-12p70 hyperproduction by IL-10$^{-/-}$ M-Mϕ. In the present study, however, we found that IL-10 also plays a novel role for the differentiation of Mϕ with anti-inflammatory phenotype. We demonstrated that abnormal IL-12p70 production by IL-10$^{-/-}$ M-Mϕ in response to stimulation with bacteria was significantly reduced by supplementation with IL-10 during the differentiation process from BM cells to M-Mϕ. Interestingly, the effect of IL-10 on the differentiation of Mϕ differs from that on the concomitant stimulation with bacteria. In the former case, only IL-12p70 production was significantly reduced, and TNF-α and IL-6 productions were suppressed just a little or not at all. In contrast, in the latter case, not only IL-12p70 but also other proinflammatory cytokines (TNF-α and IL-6) were completely suppressed. These results indicated that, in anti-inflammatory Mϕ subsets, IL-12 was regulated during the differentiation process by endogenous IL-10, but TNF-α and IL-6 were not regulated in this process. Thus, endogenous IL-10, which is induced during differentiation of M-Mϕ, functionally regulates Mϕ to acquire an anti-inflammatory phenotype such as the hypoproduction of IL-12. Moreover, abnormally differentiated BM-derived M-Mϕ and CLPMϕ in IL-10$^{-/-}$ mice may show an abnormal response to bacteria, produce extraordinary amounts of IL-12 and IL-23, and contribute to the pathogenesis of colitis in IL-10$^{-/-}$ mice. Similarly to these results, we previously demonstrated that endogenous IL-10 plays a key role in phenotype determination of M-Mϕ in humans (27, 41), and IL-10 is produced during the differentiation of monocyte-derived M-Mϕ, but not of GM-Mϕ (30). In human IBDs, monocytes obtained from some patients who have Crohn's disease did not differentiate normally with M-CSF stimulation (T. Hisamatsu, unpublished observation). This observation suggests the possibility that M-Mϕ from patients with Crohn's disease show an abnormal phenotype and contribute to the pathogenesis of intestinal inflammation.

In conclusion, results of the present study demonstrate that BM-derived M-Mϕ and intestinal Mϕ show an anti-inflammatory phenotype, which involves the production of large amounts of IL-10, but a failure to produce IL-12 and IL-23 upon stimulation with bacteria, and intestinal Mϕ may play important roles in gut homeostasis. However, IL-10 deficiency during differentiation of these Mϕ altered their characteristics into a proinflammatory phenotype, which was characterized by the production of huge amounts of IL-12 and IL-23 after bacteria recognition. Thus, these abnormal responses of intestinal Mϕ upon the bacteria may contribute to Th1 polarization, and cause chronic colitis via IL-12 and IL-23 hyperproductions. Our data provide new insights into the intestinal Mϕ to gut flora relationship in the development of colitis in IL-10$^{-/-}$ mice.

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Disclosures

The authors have no financial conflict of interest.

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Is there a role for apheresis in gastrointestinal disorders?

Toshifumi Hibi* and Atsushi Sakuraba

T Hibi is Professor of Gastroenterology and Head of the Inflammatory Bowel Disease Center, and A Sakuraba is an Instructor at the Inflammatory Bowel Disease Center of Keio University, Japan.

Correspondence
*Division of Gastroenterology
Department of Internal Medicine
School of Medicine
Keio University
35 Shinanomachi
Shinjuku
Tokyo 160-8582
Japan
thibi@sc.itc.keio.ac.jp

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APHERESIS is now a common therapeutic modality, with proven clinical benefits for several gastrointestinal disorders including inflammatory bowel disease (IBD),[1] pouchitis, intestinal Bechét's disease, alcoholic hepatitis and hepatitis C. Gastroenterologists have limited experience with apheresis, however, owing to the complexity of the process.

The etiology and pathogenesis of IBD remain obscure, but it is known that immunologic factors are involved and there is consensus that the inflamed mucosa of IBD patients are infiltrated by large numbers of inflammatory cells. The treatment of IBD with leukocyte apheresis is based on the hypothesis that removal of the circulating cells involved in intestinal inflammation will re-establish the balance between uncontrolled and controlled mucosal inflammation. The efficacy of leukocyte apheresis might not be restricted to cell removal, however, as contact activation of cells with the treatment apparatus or a change in proportions of regulatory T cells might also have immunomodulatory effects.

A single session of leukocyte apheresis removes approximately 3×10^9–10×10^9 granulocytes, which may be only ~10% of those circulating in the peripheral blood. A temporal decrease in the numbers of peripheral blood leukocytes and/or granulocytes is observed during the procedure, followed by a rapid return to the pretreatment level or higher. This rise in leukocyte numbers could be explained by the mobilization of leukocytes from the marginal pool. This results in a decrease in activated leukocytes and an increase in immature leukocytes in the peripheral blood. Under inflammatory conditions, the number and survival time of granulocytes are increased, and their serial removal could contribute to the anti-inflammatory effect of apheresis. Apheresis results in downregulation of several adhesion molecules and chemokine receptors on leukocytes, which might inhibit their migration to inflamed tissues. Furthermore, the capacity of peripheral leukocytes to produce proinflammatory cytokines is suppressed. The concomitant depletion of platelets could also contribute to their immunomodulatory effects. Thus, the overall effects of apheresis might be due to reduced levels of activated leukocytes followed by an increase in the numbers of immature leukocytes; diminished leukocyte migration to the inflamed tissue; and a reduction in the levels of proinflammatory cytokines. Studies are needed to identify the exact mode of action of apheresis.

Several devices have been developed to improve the efficiency and simplify the process of leukocyte removal. Granulocyte and monocyte adsorption apheresis (GCAP), an adsorptive type of extracorporeal apheresis, is performed with the Adacolumn® apheresis system (Japan Immunoresearch Laboratory, Japan). The device contains cellulose-coated acetate beads, and adsorbs ~65% of granulocytes, 55% of monocytes, 2% of lymphocytes and a small number of platelets from the blood. A single procedure removes a total of ~4.0×10^9 granulocytes and monocytes from the blood circulation. Leukocytapheresis (LCAP) performed with the Cellsorba® system (Asahi Medical, Japan), uses a filter consisting of nonwoven hollow fibers to trap leukocytes. LCAP nonselectively removes ~13.0×10^9 leukocytes and 5.2×10^{11} platelets from the circulating blood during one session of treatment. The removal efficacy of leukocytes passing through the filter is close to 100%. In both procedures, blood inlet and outlet are via suitable veins in the bilateral forearms of the patient and small amounts of anticoagulants are added to the circulation. Sessions are performed at a flow rate of 30–50 ml/min for 1 h and weekly for 5–10 sessions. GCAP and LCAP have both been approved by the Japanese Government for the treatment of ulcerative colitis. LCAP is also approved for the treatment of rheumatoid arthritis.

Several uncontrolled trials of plasmapheresis[2] and lymphocyte apheresis[3] have shown efficacy in active Crohn's disease; however, it has also been reported that the latter was unable to prevent relapse in those patients with corticosteroid-induced remission.[4] The results of previous studies in Crohn's disease using the centrifugal

method to remove leukocytes are controversial. Studies of GCAP and LCAP in Crohn's disease are limited and only small pilot studies have been reported. Weekly treatment with GCAP or LCAP was, however, reported to be effective in >50% of treated patients.[5]

Nationwide multicenter trials have been carried out in Japan to assess the efficacy and safety of GCAP or LCAP compared with corticosteroid treatment in patients with active ulcerative colitis refractory to conventional medication.[6,7] GCAP and LCAP showed clinical improvement in ~66% of patients, which was significantly higher than in patients treated with corticosteroids. In addition, significantly fewer adverse effects were seen in the GCAP and LCAP treatment groups; only mild adverse effects such as dizziness and nausea were observed in ~20% of the patients.

A double-blind controlled trial of LCAP showed that weekly treatment was more efficacious and safer than sham apheresis treatment; clinical improvement was shown in 80% of LCAP-treated patients compared with 33% of the sham-treated patients.[8] A significant advantage was demonstrated, but the study was rather small (10 patients per treatment group), performed for active ulcerative colitis refractory to conventional drug therapy, and most patients were also receiving simultaneous corticosteroids.

The efficacy of GCAP in patients with active ulcerative colitis who were not on concomitant corticosteroids has also been reported.[9] More than 80% of steroid-naive patients showed a response to GCAP.

Weekly GCAP or LCAP induced remission in 3–4 weeks in ~66% of patients with moderate to severe active ulcerative colitis. Moreover, only minor side effects such as nausea, fever and rashes were observed in ~20% of the patients.

We have found that triweekly treatment with GCAP is more effective than weekly treatment, and therefore shortens the time needed to achieve remission without increasing the incidence of side effects (T Hibi and A Sakuraba, unpublished data). Furthermore, in a retrospective analysis, we found that weekly GCAP therapy was effective in patients who were steroid-refractory as well as in patients with steroid-dependent ulcerative colitis, thus allowing the dosage of corticosteroids to be reduced in these patients.[10] In another preliminary study we confirmed that biweekly treatment of GCAP was as effective as 6-mercaptopurine in maintaining remission of ulcerative colitis. Several reports have also been published on the efficacy of GCAP in pediatric patients with ulcerative colitis, for whom treatment with corticosteroids should ideally be avoided.

The standard therapeutic strategies used to treat IBD are reliant on drugs and carry with them associated adverse side effects. A treatment that is highly effective and safe has been long awaited and leukocyte apheresis might be the right candidate. This treatment should be considered as a nonpharmacologic adjunct to conventional therapy, however, as a single round of GCAP or LCAP will cost nearly US$1,000. At present, its use should be limited to those patients who are steroid-refractory, or for whom corticosteroids are contraindicated.

Preliminary studies indicate that GCAP or LCAP is efficacious and safe in patients with IBD. To obtain maximum efficacy, we recommend that it should be performed triweekly for a total of 6–10 sessions. To prove the efficacy and assess the most efficient frequency of apheresis, further larger controlled studies should be conducted.

Supplementary information, in the form of a figure is available online on the *Nature Clinical Practice Gastroenterology & Hepatology* website.

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GLOSSARY

APHERESIS
The removal of blood from the body and withdrawal of a specific component. Blood is retransfused to the body once the process is complete

Competing interests
The authors declared they have no competing interests.

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Macrophage migration inhibitory factor contributes to the development of acute dextran sulphate sodium-induced colitis in Toll-like receptor 4 knockout mice

T. Ohkawara,*† H. Takeda,*
J. Nishihira,† K. Miyashita,*
M. Nihiwaki,† Y. Ishiguro,‡ K. Takeda,§
S. Akira,¶ T. Iwanaga,** T. Sugiyama††
and M. Asaka*
*Department of Gastroenterology and Hematology, Hokkaido University Graduate School of Medicine, Sapporo, Japan, †Department of Research and Development, GeneticLab Co. Ltd, Sapporo, Japan, ‡First Department of Internal Medicine, Hirosaki University School of Medicine, Hirosaki, Japan, §Division of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan, ¶Department of Host Defense, Research Institute for Microbial Disease, Osaka University, Suita, Japan, **Department of Functional Morphology, Hokkaido University Graduate School of Medicine, Sapporo, Japan, ††Third department of Internal Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan

Accepted for publication 25 May 2005
Correspondence: Dr Tatsuya Ohkawara, Department of Gastroenterology and Hematology, Hokkaido University Graduate School of Medicine, Sapporo 060–8638, Japan.
E-mail: tokawara@med.hokudai.ac.jp

Summary

Toll-like receptor 4 (TLR4), which recognizes lipopolysaccharides, plays an important role in the innate immune response. In this study, we investigated the role of TLR4 in the development of experimental colitis with regard to the biological actions of macrophage migration inhibitory factor (MIF) using TLR4 null ($^{-/-}$) mice. TLR4$^{-/-}$ mice were given 2% dextran sulphate sodium (DSS) in drinking water to induce colitis, which was clinically and histologically as severe as that seen in wild-type (WT) mice. The level of tumour necrosis factor (TNF)-α in colon tissues was increased in WT mice but unchanged in TLR4$^{-/-}$ mice. The level of myeloperoxidase (MPO) activity in colon tissues was increased by DSS administration in both TLR4$^{-/-}$ and WT mice. The expression of MIF was up-regulated in the colons of TLR4$^{-/-}$ mice with acute DSS-induced colitis. An anti-MIF antibody significantly suppressed colitis and elevation of matrix metalloproteinase-13 in TLR4$^{-/-}$ mice. The current results obtained from TLR4$^{-/-}$ mice provide evidence that MIF plays a critical role in the development of acute DSS-induced colitis.

Keywords: dextran sulphate sodium-induced colitis, inflammatory bowel disease, inhibitory factor, macrophage migration, Toll-like receptor 4

Introduction

Inflammatory bowel diseases (IBDs), including Crohn's disease (CD) and ulcerative colitis, are characterized by chronic and relapsed inflammation of the gut, but their aetiology remains unknown [1]. Recent studies have shown that various inflammatory mediators, such as tumour-necrosis factor (TNF)-α, interleukin (IL)-6 and macrophage migration inhibitory factor (MIF), are involved in the pathogenesis of IBDs [2–5]. In fact, humanized antibodies specific for TNF-α and IL-6 have been developed for treatment of patients with CD [6–8]. Recently, several studies have focused on dysfunction of the innate immune response in the pathogenesis of IBDs [1,9,10].

Toll-like receptor (TLR), the human homologue of *Drosophila* Toll, plays an essential role in the innate immune response. TLR belongs to the IL-1/Toll receptor family [11–14] and to a family of pattern-recognition receptors that detect conserved molecular products of microorganisms [15]. TLR4, one of the TLRs, is the receptor for lipopolysaccharides (LPS), the major component of a Gram-negative bacterial cell wall. Accordingly, lack of TLR4 abolishes LPS-induced inflammation and disorders, including endotoxin shock [16]. Various polymorphisms have been identified in genes encoding for TLR4 [17,18]. In the gastrointestinal tract, it has been reported that intestinal epithelial cells express some pattern-recognition receptors *in vitro* [19] and that expression of TLR4, but not TLR3, was observed in

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epithelial cells from the colons of patients with IBD [20]. In the mouse colon, it has been reported that TLR4 protein and mRNA expression were significantly up-regulated during dextran sulphate sodium (DSS)-induced colitis [21].

MIF is the first cytokine discovered in T lymphocytes [22,23]. This cytokine is expressed ubiquitously in various kinds of cells and has been re-evaluated as a multi-functional molecule involved in immune response [24,25]. MIF is known to be a proinflammatory cytokine released mainly by macrophages [26] and a T lymphocyte activator in immune responses [27]. MIF is essential for the development of LPS-induced disorders, and neutralization of MIF by its antibody protects mice and rats against endotoxin shock [28]. In the cytokine network, TNF-α plays an important role in the development of inflammation, in which MIF regulates expression of TNF-α [28–30].

In this study, we attempted to clarify further the mechanism underlying the development of DSS-induced colitis with regard to the cytokine network using TLR4 null ($^{-/-}$) mice, and particularly with respect to the relationship between MIF and TNF-α. We found that MIF was up-regulated during DSS-induced colitis independently of TNF-α through TLR4 signalling. Our results provide novel evidence that MIF plays a critical role in the development of intestinal inflammation independently of TNF-α.

Materials and methods

Materials

Nitrocellulose membrane filters were obtained from Millipore (Bedford, MA, USA). The ECL Western blotting detection system was from Amersham Bioscience (Piscataway, NJ, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody and Micro BCA protein assay kits were from Pierce (Rockford, IL, USA). DSS (molecular mass, 40 kilodaltons) and rabbit IgG fraction were from ICN Biomedicals (Aurora, OH, USA), and anti-β-actin antibody was from Sigma-Aldrich Co. (Temecula, CA, USA). Enzyme-linked immunosorbent assay (ELISA) development kits for mouse TNF-α were from Genzyme Techne (Minneapolis, MN, USA). Recombinant mouse MIF and a polyclonal anti-MIF antibody were prepared as described previously [31,32]. All other chemicals used were of analytical grade.

Mice

Specific pathogen-free C57BL6 male mice 8–10 weeks of age were purchased from Japan Charles River Co. (Shizuoka, Japan). TLR4$^{-/-}$ mice (background: C57BL) were established as described previously [16]. Mice were inbred under a light : dark cycle every 12 h at room temperature and fed food and water *ad libitum*. The experimental protocol adhered to the Declaration of Helsinki and was in accordance with the Animal Experiment Ethics Committee of the Graduate School of Medicine of Hokkaido University.

Induction and general assessment of DSS colitis

To induce colitis, mice were given 2% DSS in distilled water *ad libitum*. Mice were weighed daily and inspected visually for rectal bleeding and diarrhoea. Mice were anaesthetized by intraperitoneal injection of thiopental on day 7 after the first administration of DSS and killed for histological evaluation and molecular analysis. The colons were removed, collected and stored at −80°C until use for molecular analysis. For histological evaluation, the samples of colons were opened longitudinally and fixed with 10% neutral buffered formalin. The disease activity index (DAI) was used to assess the grade of colitis based on a previously published scoring system by Cooper *et al.* [33]. The DAI score correlates well with tissue damage scores and with inflammatory mediators such as myeloperoxidase (MPO) activity. To exclude bias, the DAI score and body weight were determined in a blinded fashion by an examiner.

Histological examination and immunohistochemistry

The colon tissues fixed with 10% neutral buffered formalin were embedded in paraffin. Sections (4 μm) were stained with haematoxylin and eosin (H&E). For histological evaluation of colitis, specimens were quantified microscopically as described previously [5]. The colocecal damage was categorized into six grades: grade 0, normal mucosa; grade 1, infiltration of inflammatory cells; grade 2, shortening of the crypt by less than half; grade 3, shortening of the crypt by more than half; grade 4, crypt loss; and grade 5, destruction of epithelial cells (ulceration and erosion). In addition, we evaluated the extent of inflammatory lesions. The extent of lesions in the total colon was classified into six grades: grade 0, 0%; grade 1, 1–20%; grade 2, 21–40%; grade 3, 41–61%; grade 4, 61–80%; and grade 5, 81–100%. To exclude bias, the histological score was determined in a blinded fashion by two pathologists.

For assessment of immunohistochemistry for MIF, the sections of tissues (5 μm) were stained with polyclonal anti-MIF antibody (diluted 100 : 1) as described previously [5].

Enzyme-linked immunosorbent assay

Colon tissue samples in phosphate buffered saline (PBS) containing a cocktail of protease inhibitors (1 μl to 20 mg of tissue according to the manufacturer's protocol) were homogenized with a Polytron homogenizer (Kinematica, Luzern, Switzerland) and centrifuged at 12 000 *g* for 10 min. The supernatants were subjected to the assay. TNF-α contents in tissues were measured using ELISA kits in accordance with the manufacturer's protocol.

Measurement of myeloperoxidase activity

Tissue MPO activity was determined by a standard enzymatic procedure as described previously [34], with minor modifications. Briefly, after the samples had been weighed, a tissue sample (approximately 300 mg) was homogenized in a buffer (0·5% hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer, pH 6·0) using a Polytron-type homogenizer three times for 30 s each on ice. The sample was centrifuged at 20 000 *g* for 20 min at 4°C and the supernatant was collected. The supernatant (100 µl) was then added to 2·9 ml of 50 mM phosphate buffer (pH 6·0) containing 0·167 mg/ml O-dianisidine hydrochloride and 0·0005% hydrogen peroxide, and absorbances were measured using a spectrometer at 25°C. The protein concentration of the supernatant was determined using a Bradford assay kit (Bio-Rad Laboratories, Hercules, CA, USA) for calibration, and the values were standardized using MPO purified from human leucocytes (Sigma, St Louis, MO, USA).

Western blot analysis

Western blot analysis for MIF was performed in accordance with a previous report [31]. Briefly, colon tissue was disrupted with a Polytron homogenizer (Kinematica, Lucerne, Switzerland). The protein concentrations of the tissue homogenates were quantified using a Micro BCA protein assay reagent kit. Equal amounts of proteins were dissolved in 20 µl of Tris-HCL, 50 mM (pH 6·8), containing 2-mercaptoethanol (1%), sodium dodecyl sulphate (SDS) (2%), glycerol (20%) and bromophenol blue (0·04%), and the samples were heated at 100°C for 5 min. The samples were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically onto nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in PBS, probed with a polyclonal anti-MIF antibody (diluted 2000 : 1) and reacted with a goat anti-rabbit IgG antibody coupled with horseradish peroxidase (HRP). Western blot analysis of matrix metalloproteinase (MMP)-13 was performed in a similar manner using an anti-MMP-13 antibody (diluted 5000 : 1; Chemicon, Temecula, CA, USA). The resultant complexes were processed for detection by an ECL Western blotting detection system according to the manufacturer's protocol. The proteins were visualized with a Konica HRP-1000 immunostaining kit in accordance with the manufacturer's protocol.

Treatment with anti-MIF antibody

Polyclonal anti-MIF antibody (0·4 mg/mouse) or non-immune rabbit IgG (0·4 mg/mouse in PBS) was injected intraperitoneally on 2, 4 and 6 days after the first DSS treatment. DAI and histology were assessed to evaluate the effect of anti-MIF antibody on DSS-induced colitis in TLR4$^{-/-}$ mice. DAI and histological scores were quantified by two pathologists in a blind fashion using a scoring system as previously described [5].

Statistics

Data are presented as the means ± standard error (SE). The results were analysed statistically using the unpaired Student's *t*-test and one-way ANOVA with a *post hoc* test (StatView; SAS Institute, Cary, NC, USA). *P*-values < 0·05 were considered to be statistically significant.

Results

Development of DSS-induced colitis in both TLR4$^{-/-}$ and WT mice

We assessed clinical symptoms using scoring systems that have been reported to be reliable indicators of pathological changes. The disease activity index, which reflects the frequencies of diarrhea, rectal bleeding and body weight loss, had increased in both TLR4$^{-/-}$ and WT mice 7 days after DSS treatment (3·5 ± 0·1 and 3·2 ± 0·3, respectively) (Fig. 1a). The colon length, which is shortened by DSS treatment, has often been used as a morphological parameter for the degree of colitis induced by DSS. Under normal conditions, no significant difference in colon length was found between TLR4$^{-/-}$ and WT mice (8·0 ± 0·1 and 8·1 ± 0·3, respectively). However, when mice were treated with 2% DSS for 7 days, the colon length was significantly shortened in TLR4$^{-/-}$ mice (6·1 ± 0·3 cm) as well as in WT mice (5·5 ± 0·2 cm) (Fig. 1b).

Histological findings in the colons of DSS-treated mice were examined to evaluate the severity of tissue damage using H&E staining. In the colons of TLR4$^{-/-}$ mice, inflammatory infiltration was minimal before DSS treatment but had increased on day 7 (Fig. 2c,d, respectively). The findings in the colons of WT mice without or with DSS-induced colitis were similar to those in TLR4$^{-/-}$ mice (Fig. 2a,b, respectively). Histological scores for tissue damage and extent of lesion in TLR4$^{-/-}$ mice were as severe as those in WT mice on day 7 (tissue damage: 4·2 ± 0·2 and 4·4 ± 0·3, respectively; extent of lesion: 2·6 ± 0·3 and 3·0 ± 0·3, respectively) (Fig. 3).

The TNF-α level was not increased in the colons of TLR4-deficient mice treated with DSS

ELISA was used to determine the level of TNF-α in the colons of TLR4$^{-/-}$ mice. The levels of TNF-α in WT and TLR4$^{-/-}$ mice before DSS treatment were similar (5·4 ± 0·9 and 2·8 ± 0·6, respectively). This level was significantly higher in the colons of WT mice with DSS colitis than in the colons of non-treated WT mice (16·7 ± 2·4) (Fig. 4). In contrast, the level of TNF-α

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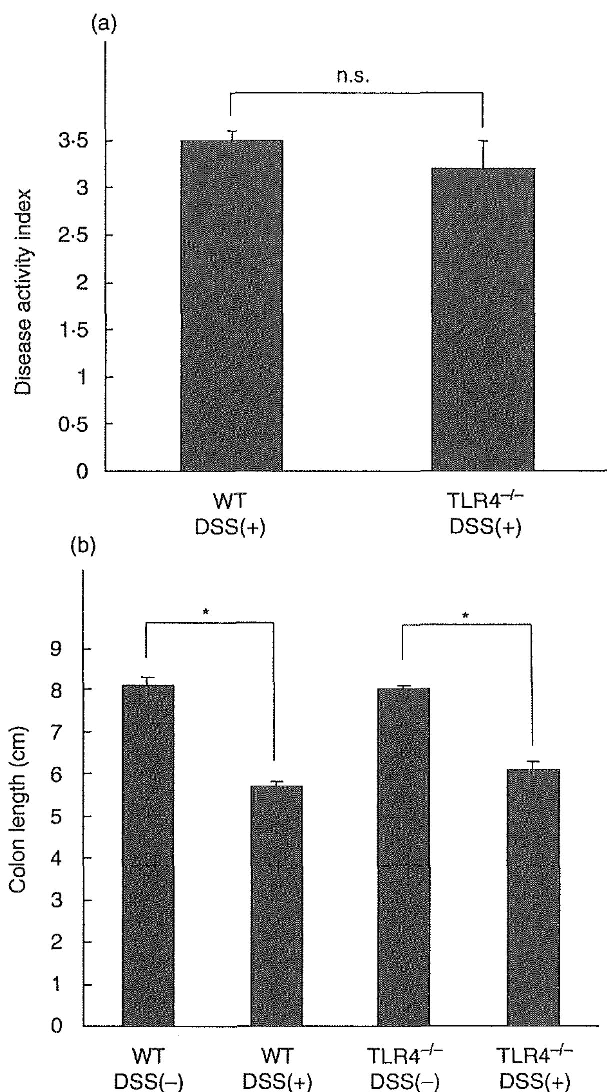

Fig. 1. Development of dextran sulphate sodium (DSS)-induced colitis in Toll-like receptor 4 null ($^{-/-}$) mice. Mice were given 2% DSS in drinking water for 7 days. (a) The disease activity index (DAI) on day 7 is expressed as the mean ± standard error (SE) of 10 mice in each group. (b) Colon length was measured 7 days after the first DSS treatment. Data are shown as the means ± SE of 10 mice in each group. *$P < 0{\cdot}05$ compared with non-treated wild-type mice.

did not increase in TLR4$^{-/-}$ mice even when colitis was induced by DSS treatment for 7 days (3·3 ± 0·4) (Fig. 4).

Myeloperoxidase activity increased in the colons of TLR4 $^{-/-}$ mice with DSS colitis

MPO activity is a marker of neutrophil contents and is up-regulated in the tissue under inflammatory conditions. We measured MPO activities in the colons of the mice. In both WT and TLR4$^{-/-}$ mice, the level of MPO activity was low before DSS treatment (1·6 ± 0·6 and 1·9 ± 0·9 U/g protein, respectively) (Fig. 5). Seven days after the first DSS treatment, the level of MPO activity in the colon was remarkably increased in both WT and TLR4$^{-/-}$ mice (6·2 ± 0·5 and 5·8 ± 0·7 U/g protein, respectively) (Fig. 5). There was no statistically significant difference between the levels of MPO activity in WT and TLR4$^{-/-}$ mice treated with DSS.

MIF expression was up-regulated in the colons of TLR4$^{-/-}$ mice with DSS colitis

To clarify the reason why DSS-induced colitis occurred in TLR4$^{-/-}$ mice, Western blot analysis for macrophage migration inhibitory factor (MIF) in the colon was performed. We have previously demonstrated up-regulation and localization of MIF in mice with DSS-induced colitis [5]. In the present study, MIF was expressed in the colons of WT and TLR4$^{-/-}$ mice without DSS treatment (Fig. 6). On the other hand, the expression of MIF was enhanced in the colons of both TLR4$^{-/-}$ and WT mice when mice were given DSS for 7 days (Fig. 6). To assess the localization of MIF protein in the colon, immunohistochemical analysis for MIF in the colons of TLR4$^{-/-}$ mice was carried out. Faint MIF-positive staining spots were seen in epithelial and mononuclear cells of mice without DSS colitis (Fig. 7b). This staining was observed clearly in infiltrating inflammatory cells in the colons of mice with DSS colitis (Fig. 7c). This staining was similar to that in WT mice (data not shown).

Anti-MIF antibody ameliorates DSS-induced colitis in TLR4$^{-/-}$ mice

To assess further the contribution of MIF to inflammation in TLR4$^{-/-}$ mice, we investigated whether blockade of MIF using an anti-MIF antibody would ameliorate colitis induced by DSS in TLR4$^{-/-}$ mice. Treatment with an anti-MIF antibody resulted in a significant reduction in DAI on day 7 ($P < 0{\cdot}01$, 1·1 ± 0·1 and 2·3 ± 0·3) (Fig. 8a) and in the histological score in TLR4$^{-/-}$ mice given DSS for 7 days compared with treatment with non-immune IgG (tissue damage: $P < 0{\cdot}05$, 1·8 ± 0·6 and 4·0 ± 0·5, respectively; extent of lesion: $P < 0{\cdot}05$, 1·2 ± 0·2 and 2·6 ± 0·4, respectively) (Fig. 8b).

MMP-13 expression was modulated by MIF in the colons of TLR4$^{-/-}$ mice with DSS-induced colitis

MMP is thought to be an important molecule in tissue destruction and remodelling. Our previous study showed that MIF modulated the expression of MMP-13 mRNA and that blockade of MIF bioactivity reduced the expression of MMP-13 mRNA. Regulation of MMP by MIF has been reported in several experimental diseases, including models of arthritis and colitis [5,35]. Western blot analysis revealed that the expression of MMP-13 was remarkably (more than twofold density in blotted bands) increased in colons of TLR4$^{-/-}$ mice treated with DSS for 7 days (Fig. 9). Moreover, treatment with an anti-MIF antibody markedly (less than

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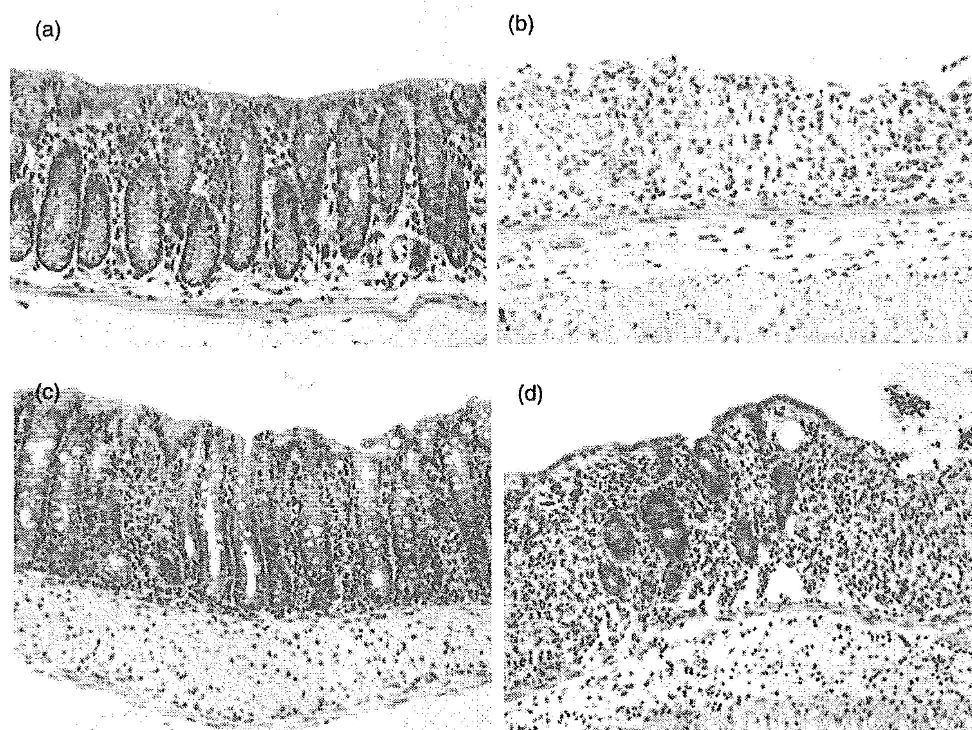

Fig. 2. Representative microphotograph of the histological appearance of the colon from TLR4$^{-/-}$ and wild-type (WT) mice with or without dextran sulphate sodium (DSS) colitis. (a) Normal colon in WT mice; (b) inflamed colon in WT mice with DSS-induced colitis; (c) colon in TLR4$^{-/-}$ mice without DSS treatment; (d) inflamed colon in TLR4$^{-/-}$ mice with DSS-induced colitis. After 7 days of DSS treatment, severe infiltrating inflammatory cells and tissue destruction occurred in the colon of both TLR4 and WT mice. Similar appearances were obtained from the colon tissues of other mice. Original magnification ×200.

half of density in blotted band) suppressed the expression of MMP-13 in the colons of TLR4$^{-/-}$ mice given 2% DSS (Fig. 9).

Discussion

An animal model of DSS-induced colitis established by Okayasu *et al.* [36] has been used widely for investigation of the pathogenesis of IBD and for assessment of novel treatments. Several inflammatory mediators such as cytokines have been shown to play an important role in the development of DSS-induced colitis [5,37–39].

In this study, we found that acute DSS-induced colitis occurred in TLR4$^{-/-}$ mice as well as in WT mice. During the course of acute DSS-induced colitis, the levels of MIF expressions were remarkably up-regulated in the colons obtained from WT and TLR4$^{-/-}$ mice. On the other hand, the production of TNF-α in colon tissues from WT mice was significantly increased in response to DSS, whereas that in TLR4$^{-/-}$ mice was minimal.

In innate immune response, TLR has recently been thought to play a central role [15], and innate immunity has been reported to play an important role in the development of DSS-induced colitis [4,40]. In the colon, Ortega-Cava *et al.* [21] have reported the expression and localization of TLR4 in the mouse gastrointestinal tract. The expression of TLR4 was observed in epithelial cells and lamina propria mononuclear cells in the normal mouse gut, including the small intestine and colon. The level of TLR4 mRNA expression was higher in the colon than in other tissues in WT mice. These findings suggested the possibility that mice lacking TLR4 would show different features of DSS-induced colitis, which prompted us investigate the DSS-induced colitis in TLR4$^{-/-}$ mice.

In this study, however, we found that severe colitis occurred in TLR4$^{-/-}$ mice treated with DSS. In addition to clinical and histological severity, the level of MPO activity, as a parameter of neutrophil accumulation, was increased in colons of TLR4$^{-/-}$ mice with DSS-induced colitis. In contrast, the increase of TNF-α level was essentially unchanged in the colons of TLR4$^{-/-}$ mice by DSS administration. The C3H/HeJ mouse strain, which is characterized by hyporesponsiveness to LPS and mutation in the TLR4 gene, showed severe colitis in response to DSS stimulation [41]. Very recently, it has been reported that MyD88 knockout mice show severe colitis induced by DSS, suggesting that TLR signalling is not essential in the development of DSS-induced colitis [42]. MyD88 is the adaptor protein associated with TLR signalling. Lack of MyD88 increased infiltration of macrophage and T cells in

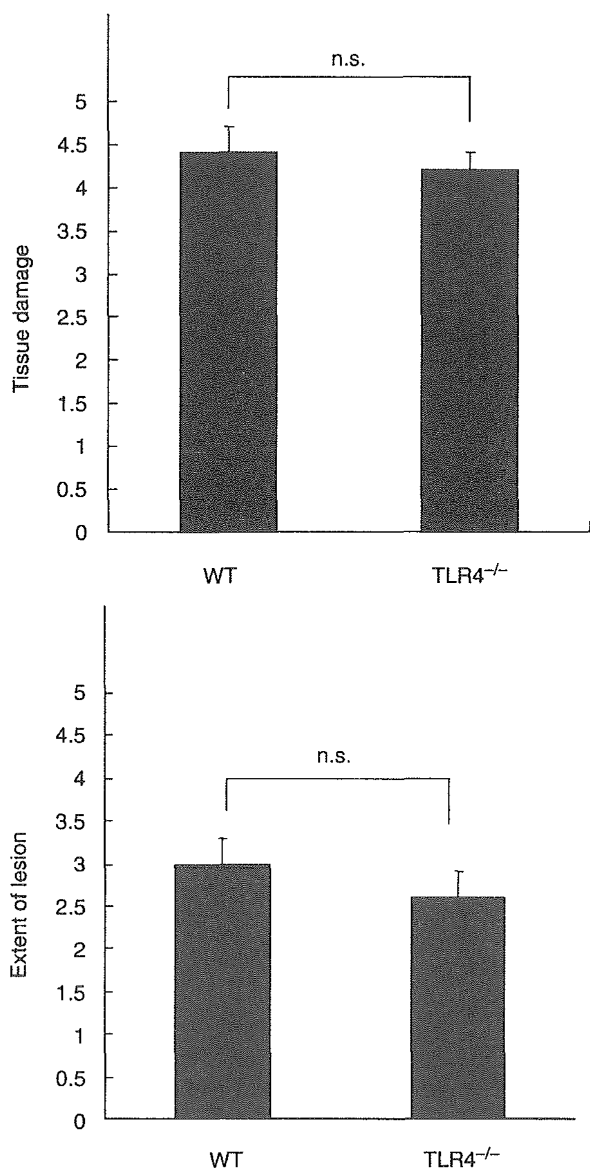

Fig. 3. Histological evaluation in the colons of mice with dextran sulphate sodium (DSS)-induced colitis. Mice were given 2% DSS in drinking water for 7 days. Histological scores of tissue damage and extent of lesion in colon tissues. Histological scores are microscopically quantified by the method described in Materials and methods. Data are expressed as the means ± SE of five mice in each group.

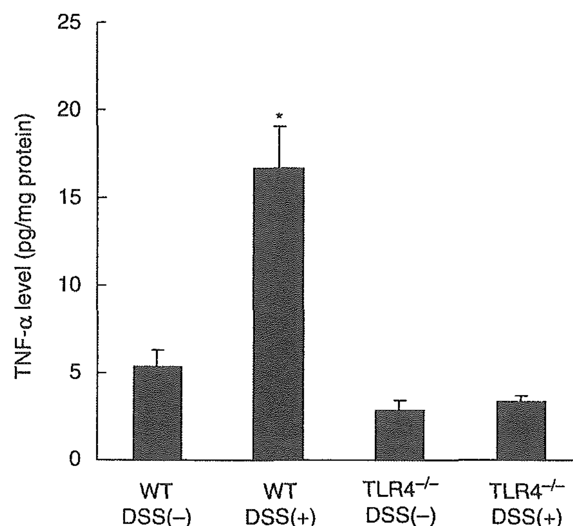

Fig. 4. Tumour necrosis factor (TNF)-α contents in the colons of mice with dextran sulphate sodium (DSS)-induced colitis. The levels of TNF-α in the colons of TLR4$^{-/-}$ mice ($n = 5$) and wild-type (WT) mice ($n = 5$) before and 7 days after DSS treatment were measured by enzyme-linked immunosorbent assay (ELISA). Data are expressed as the means ± SE of five mice in each group. *$P < 0{\cdot}05$ compared with non-treated WT mice.

the colon of mice with DSS-induced colitis. Consistent with these findings, our results also indicate that TLR4 may not play a central role in the development of DSS-induced colitis. Furthermore, our data suggest that MIF induces inflammation under conditions of lacking TLR4.

In general, TNF-α plays an important role in inflammation and immune response. Several studies have shown the role of TNF-α in human IBD and experimental colitis [1–3,42]. On the other hand, MIF and TNF-α have been shown to have a close relationship in various events in inflammatory processes *in vivo* and *in vitro* [5,28–30,43]. In our previous study, we found that WT mice with colitis induced by 7 days of DSS treatment showed increases in both TNF-α and MIF expression levels in the colon [5]. For example, Kobayashi *et al.* also demonstrated that up-regulation of the

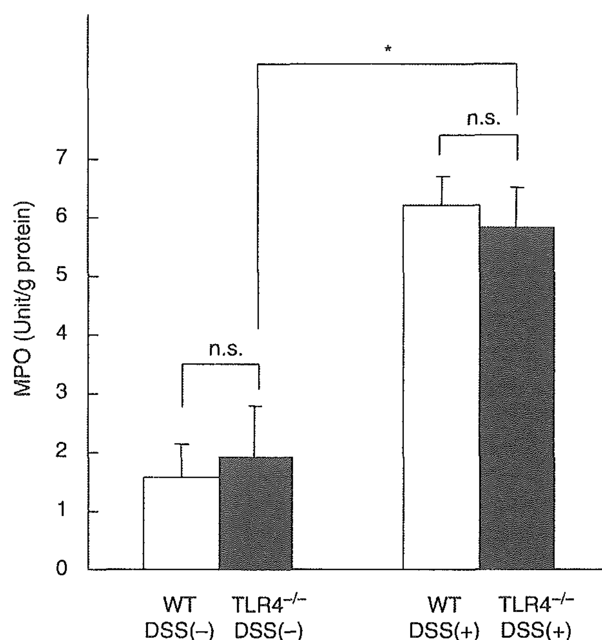

Fig. 5. Myeloperoxidase (MPO) activity in the colons of mice given 2% dextran sulphate sodium (DSS) for 7 days. Mice were given 2% DSS in drinking water for 7 days. The levels of MPO activities were measured in colon tissues from mice on day 7. Data are expressed as the means ± SE of five mice in each group. *$P < 0{\cdot}05$.

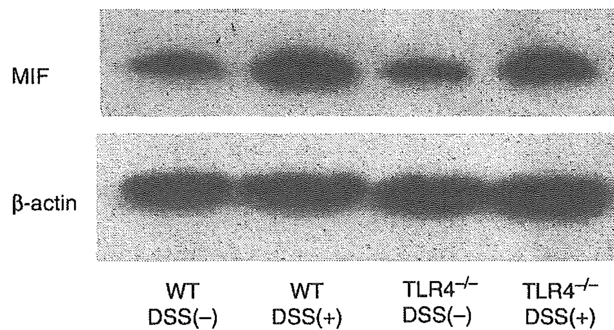

Fig. 6. Up-regulation of macrophage migration inhibitory factor (MIF) expression in the colons of TLR4$^{-/-}$ mice given dextran sulphate sodium (DSS) for 7 days. The samples of colon tissues were removed from wild-type and TLR4$^{-/-}$ mice before and 7 days after the first DSS treatment. The expression of MIF in the colons was assessed by Western blot analysis. β-actin is used as a loading control. Similar results were obtained from the three experiments.

MIF level occurred earlier than that of the TNF-α level in a model of LPS-induced hepatitis [29]. Roger *et al.* also have shown that MIF mainly regulates TNF-α through TLR4 pathway *in vitro* [44]. In this study, TNF-α levels in colon tissues from TLR4$^{-/-}$ mice were not increased during acute DSS-induced colitis. Our results also suggest the possibility that MIF regulates TNF-α expression through the TLR4 signalling pathway in this model. However, it has been reported that chronic but not acute DSS-induced colitis model is known to be associated with TNF-α [46]. Moreover, it has been reported previously that the absence of TNF-α did not suppress the severity of acute DSS-induced colitis [45]. In this study, we did not investigate the expression of TNF-α in the colon in chronic DSS-induced colitis. Thus, our current results did not completely provide the evidence that the development of DSS-induced colitis is independent of TNF-α expression. Further study is needed for elucidation of the precise role of TNF-α in DSS-induced colitis.

We have demonstrated previously that MIF plays an important role in the development of DSS-induced colitis in mice [5,39]. MIF is expressed constitutively in the gastrointestinal tissues *in vivo* and *in vitro* [4,5,47–49]. de Yong *et al.* have focused on the pathological role of MIF in chronic colitis, revealing its pathogenic role in the disease [4]. In this study, we first investigated the expression of MIF in the colon of TLR4$^{-/-}$ mice. Interestingly, the expression of MIF was significantly up-regulated in the colon of TLR4$^{-/-}$ mice with DSS-induced colitis. This result indicates that MIF is a critical component in the colon during DSS-induced colitis, independently of TLR4 signalling.

Several studies have revealed the localization of MIF-positive staining in various tissues, such as immune cells and epithelial cells in skin with dermatitis [5,31,32,50]. Similar to the findings in skin [31], we previously observed MIF expression in epithelial cells and infiltrating immune cells of the mouse colon [5]. Consistent with these findings, we here observed weak MIF expression in mucosal intestinal epithelial cells and mononuclear cells of the colon from TLR4$^{-/-}$ mice under normal conditions. MIF-positive staining was greatly enhanced in numerous infiltrating inflammatory

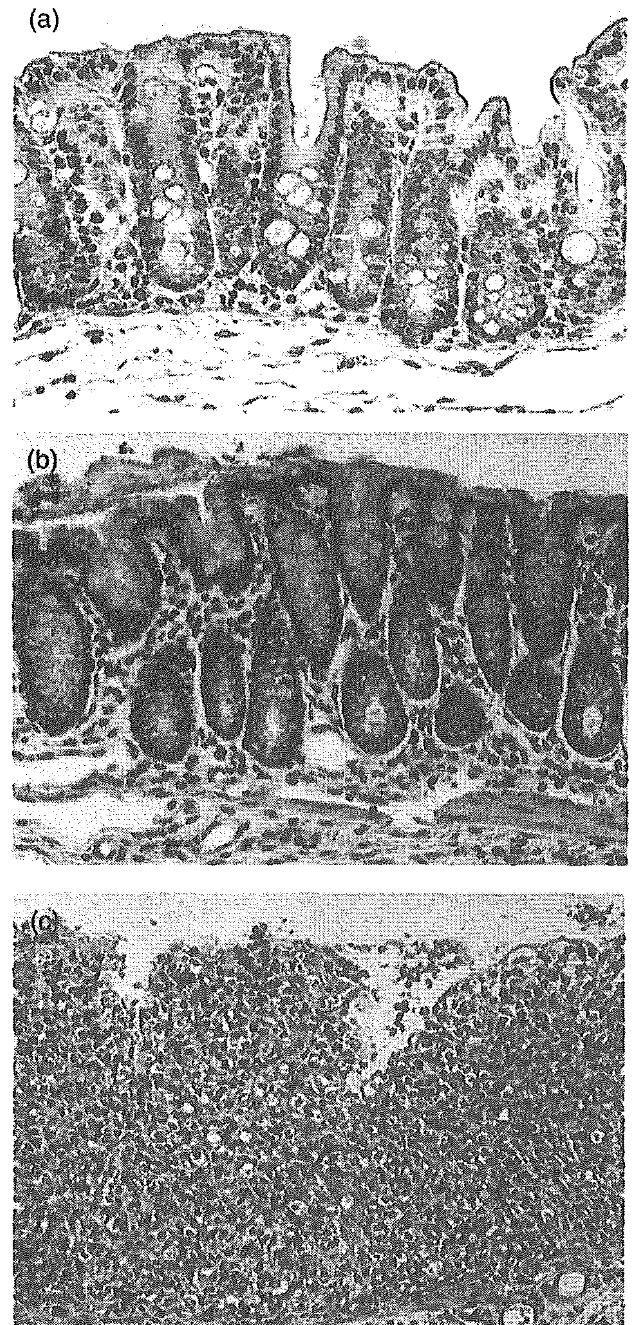

Fig. 7. Localization of macrophage migration inhibitory factor (MIF)-positive cells in the colon. Sections of colons from TLR4$^{-/-}$ mice were stained with anti-MIF antibody (diluted 100 : 1). (a) A section stained with rabbit IgG as the primary antibody (negative control). (b) A section of the colon from non -treated mice. (c) A section of the colon from mice with colitis induced by DSS for 7 days. Original magnification ×200. The sections shown are typical of the other samples.

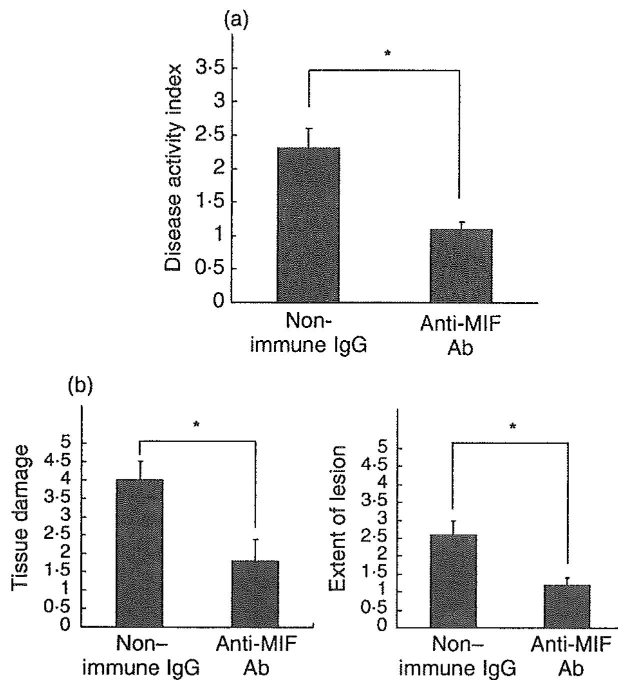

Fig. 8. Ameliorating effect of anti-macrophage migration inhibitory factor (MIF) antibody on dextran sulphate sodium (DSS)-induced colitis in TLR4$^{-/-}$ mice. TLR4$^{-/-}$ mice were treated with anti-MIF antibody or non-immune IgG (0·4 mg/time/mouse) 2, 4 and 6 days after the first DSS treatment. (a) DAI values on day 7 are expressed as the means ± SE of five mice in each group. *$P < 0{\cdot}05$ compared with non-immune IgG-treated mice. (b) Histological scores of tissue damage and extent of lesion in the colon. Data are expressed as the means ± SE of five mice in each group. *$P < 0{\cdot}05$ compared with non-immune IgG-treated mice.

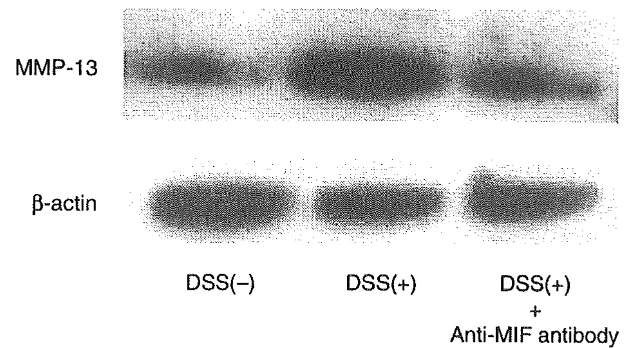

Fig. 9. Suppressive effect of anti-macrophage migration inhibitory factor (MIF) antibody on up-regulation of matrix metalloproteinase (MMP)-13 expression in the colons of TLR4$^{-/-}$ mice with dextran sulphate sodium (DSS)-induced colitis. Mice were treated with anti-MIF antibody or non-immune IgG (0·4 mg/time/mouse) 2, 4 and 6 days after the first administration of 2% DSS. Colon tissue samples were removed from non-treated, non-immune IgG- and DSS-treated, and anti-MIF antibody- and DSS-treated mice on day 7. The expression of MMP-13 in the colons was assessed by Western blot analysis. β-actin was used as a loading control. Similar results were confirmed in more than three experiments.

cells in the colonic mucosa of TLR4$^{-/-}$ mice with DSS-induced colitis. These facts provide additional evidence that local MIF expression contributes to inflammatory responses in TLR4$^{-/-}$ mice with DSS-induced colitis.

To examine further the role of MIF in the development of DSS-induced colitis in TLR4$^{-/-}$ mice, we evaluated MPO as a parameter of neutrophil accumulation. Makita *et al.* reported an increase of MPO activity and up-regulation of MIF expression in alveoli from mice with experimental acute lung distress syndrome [51]. In this study, the level of MPO activity was increased markedly in both WT and TLR4$^{-/-}$ mice with DSS-induced colitis. Thus, it is conceivable that MIF enhances the accumulation and activation of neutrophils in colons of mice with DSS-induced colitis.

Alternatively, MIF is known to up-regulate expression of MMP in cells and tissues [35]. MMP is an important molecule in tissue destruction and remodelling. We have demonstrated previously that neutralization of MIF by anti-MIF antibody suppressed the MMP-13 mRNA level, which was up-regulated in the colons of mice with DSS-induced colitis [5]. Consistent with these findings, we here found that TLR4$^{-/-}$ mice given DSS showed up-regulation of MIF and MMP-13 expressions in the colon. Moreover, anti-MIF antibody remarkably suppressed the up-regulation of MMP-13 expression in the colons of TLR4$^{-/-}$ mice with DSS-induced colitis. These facts indicate that expression of MMP-13 is modulated by MIF, which promotes DSS-induced colitis even in TLR4$^{-/-}$ mice. That is, MIF may enhance tissue damage through up-regulation of MMP-13 in the colons of TLR4$^{-/-}$ mice with DSS-induced colitis.

In conclusion, we have demonstrated that colitis could be induced by DSS in TLR4$^{-/-}$ mice. Our current results indicate that MIF may play a more crucial role than previously thought in the development of DSS-induced colitis independently of activation of innate immune responses mediated by the TLR4-signalling pathway.

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Hepatocyte Growth Factor Facilitates the Repair of Large Colonic Ulcers in 2,4,6-Trinitrobenzene Sulfonic Acid–Induced Colitis in Rats

Masatsugu Numata, MD,† Akio Ido, MD,† Akihiro Moriuchi, MD,† Ildeok Kim, MD,† Yoshihiro Tahara, MD,* Shojiro Yamamoto, MD,* Satoru Hasuike, MD,* Kenji Nagata, MD,* Yoshifumi Miyata, MD,* Hirofumi Uto, MD,* and Hirohito Tsubouchi, MD*†*

Background: Hepatocyte growth factor (HGF) modulates intestinal epithelial cell proliferation and migration, serving as a critical regulator of intestinal wound healing. The aim of this study was to clarify the effects of administration of recombinant human HGF on colonic mucosal damage in vivo.

Methods: Rats were given 7.5 mg of 2,4,6-trinitrobenzene sulfonic acid (TNBS) per rectum on day 0. On day 5, the degree of TNBS-induced colitis was evaluated endoscopically, and rats suffering from large ulcers (occupying more than two thirds of the luminal circumference) were treated with intravenous bolus injections of recombinant human HGF (1.0 mg/kg per day) or phosphate-buffered saline (PBS) for 5 days.

Results: Rats with TNBS-induced colitis given human HGF showed a significant reduction in colonic ulcer coverage and large intestinal shortening compared with those treated with PBS. Administration of recombinant human HGF also stimulated the proliferation of epithelial cells and reduced the inflammatory cell infiltrate. Finally, HGF treatment decreased the myeloperoxidase activity and tumor necrosis factor α levels in the TNBS-inflamed colon tissues.

Conclusions: These results indicate that intravenous injection of HGF accelerates colonic mucosal repair and reduces infiltration of inflammatory cells in rats with TNBS-induced colitis and suggest that HGF has the potential to be a new therapeutic modality to promote intestinal mucosal repair in patients with inflammatory bowel disease.

Key Words: 2,4,6-trinitrobenzene sulfonic acid, hepatocyte growth factor, inflammatory bowel disease, mucosal injury, mucosal repair

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The mucosal lining of the intestinal tract is composed of a rapidly proliferating and continually renewing sheet of epithelial cells. After mucosal injury occurs, numerous growth factors and cytokines, induced in both the lumen and in submucosal locations, cooperatively stimulate epithelial mucosal repair.[1–3]

Hepatocyte growth factor (HGF) was originally purified from the plasma of patients with fulminant hepatic failure and is a major agent promoting hepatocyte proliferation.[4,5] HGF also functions as a pleiotropic factor, acting as a mitogen, morphogen, and motogen for multiple subsets of epithelial cells, including gastrointestinal epithelial cells.[4–9] HGF acts primarily by ligating the c-Met receptor at the plasma membrane.[10] Recent studies have shown that HGF expression is stimulated in inflamed colonic mucosal tissue in patients with ulcerative colitis and that plasma HGF levels are increased in animal models of acute colitis.[11–13] Additionally, 2 HGF-associated molecules involved in the activation of HGF in injured tissues, HGF activator and HGF activator inhibitor type-1, are closely associated with colonic mucosal repair.[14,15] These findings indicate an important role for HGF in intestinal mucosal wound healing.

The primary therapies for inflammatory bowel disease (IBD) are anti-inflammatory and anti-immune agents such as salazosulfapyridine, mesalazine, corticosteroids, azathioprine, 6-mercaptopurine, methotrexate, and cyclosporine.[16] A chimeric mouse-human monoclonal antibody against tumor necrosis factor α (TNFα) has also been developed and has been extremely effective in Crohn's disease.[17] The majority of patients with IBD benefit from these anti-inflammatory and/or anti-immune agents, but the disease is often recurrent and intractable. We recently reported that continuous intraperitoneal administration of recombinant human HGF, which results in detectable serum levels of human HGF, facilitated colonic mucosal repair

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From the *Department of Internal Medicine II, Faculty of Medicine, University of Miyazaki, Kiyotake, Miyazaki, Japan; and †Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, Kyoto, Japan.
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Reprints: Hirohito Tsubouchi, Department of Internal Medicine II, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki 889-1601, Japan (e-mail: htsubo@med.miyazaki-u.ac.jp)
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in experimental ulcerative colitis in rats.[18] Recombinant human HGF will soon be available for treatment of patients with severe liver disease. Therefore, in contrast to various other therapeutic agents used to suppress inflammation and immunity, HGF has the potential to be an important new modality for the promotion of intestinal mucosal repair in patients with IBDs. However, intravenously administered recombinant human HGF disappears rapidly from serum because of its short half-life,[19] and it is still not clear whether intravenous injection of recombinant HGF can facilitate wound healing of deeper and more extensive mucosal damage. In this study, we used a rat model of experimental colitis induced by a single enema of 2,4,6-trinitrobenzene sulfonic acid (TNBS) and performed colonoscopies in all rats given TNBS to confirm the radial extent of mucosal damage. Only animals suffering from large colonic ulcers were treated by intravenous bolus injections of recombinant human HGF, and we evaluated the effect of this HGF treatment on wound healing and inflammation.

MATERIALS AND METHODS

Animals

Male Wistar rats, 7 weeks of age and weighing between 140 and 150 g, were obtained from Japan SLC (Shizuoka, Japan). The animals were maintained under constant room temperature (25°C) and given free access to water and a standard diet throughout the study. The protocol for animal studies was approved by the ethical committee of the Graduate School of Medicine, Kyoto University (Kyoto, Japan). All animal experiments were performed after a 1-week acclimation period. Colitis was induced in rats by administration of 7.5 mg of TNBS (Wako Pure Chemical Industries, Osaka, Japan) per rectum, dissolved in 0.5 mL of 50% ethanol (day 0). To evaluate the severity of colitis, the rats' body masses and disease activity index (DAI) scores were examined on days 1, 3, 5, 7, and 10.[20] Rats were killed on day 10, and we measured the length of the large intestine between the colocecal junction and the anal verge.

Colonoscopy

Colonoscopic examinations were performed using a BF3C40 bronchofiberscope (Olympus Co., Tokyo, Japan), on days 5 and 10. The endoscope was inserted through the anus of rats with TNBS-induced colitis under the inhalation anesthesia of diethyl ether. When the endoscope was fully inserted (~8 cm), it was possible to observe at least two thirds of the entire colon.

Administration of Recombinant Human HGF

Recombinant human HGF was kindly provided by Mitsubishi Pharma Co. (Tokyo, Japan). After endoscopic examination on day 5, either recombinant human HGF (1.0 mg/kg) in phosphate-buffered saline (PBS) or PBS alone was intravenously injected in a bolus to TNBS-induced colitis rats for 5 days (from days 5 to 9).

Measurement of the Area of Colonic Erosions

The large intestines of treated rats were obtained on day 10 and opened longitudinally. After measurement of colon lengths from the colocecal junction to the anal verge, large intestines were fixed with 10% formalin for 3 days. The areas of colonic ulcers were measured using a VM-30 micrometer (Olympus Co.).

Histologic Examination

As described above, the entire colon was excised postmortem and fixed with 10% formalin for histologic analysis. The longitudinal sections were embedded in paraffin and stained with hematoxylin and eosin (H&E). Histologic scoring was assessed independently by 2 investigators blinded to the previous protocols, using the following histopathological grading system: grade 0, normal findings; grade 1, mild mucosal and/or submucosal inflammatory infiltrate (admixture of neutrophils) and edema, punctuate mucosal erosions associated with capillary proliferation, but muscularis mucosa intact; grade 2, grade 1 changes involving 50% of the specimen; grade 3, prominent inflammatory infiltrate and edema (neutrophils usually predominating), frequently with deeper areas of ulceration extending through the muscularis mucosae into the submucosa, and rare inflammatory cells invading the muscularis propriae but without muscle necrosis; grade 4, grade 3 changes involving 50% of the specimen; grade 5, extensive ulceration with coagulative necrosis bordered inferiorly by numerous neutrophils and lesser numbers of mononuclear cells, and necrosis that extends deeply into the muscularis propria; grade 6, grade 5 changes involving 50% of the specimen.[21,22]

Myeloperoxidase Assay

Myeloperoxidase (MPO) activity in the colon tissues, which is directly related to the number and activity of infiltrating myeloid cells, was measured as described previously.[23,24] Colonic MPO activity is expressed in units per gram of wet tissue.

Immunohistochemistry

To evaluate proliferation of the colonic epithelium, 50 mg/kg of 5-bromo-2′-deoxyuridine (BrdU; Wako Pure Chemical Industries) was injected intraperitoneally 1 hour before death, and cells synthesizing DNA were identified by immunohistochemistry using a BrdU In-Situ Detection Kit (BD Bioscience, San Diego, Calif.) according to the manufacturer's instructions. We then counted the BrdU-positive

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