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Abnormally Differentiated Subsets of Intestinal Macrophage Play a Key Role in Th1-Dominant Chronic Colitis through Excess Production of IL-12 and IL-23 in Response to Bacteria[1]

Nobuhiko Kamada,* Tadakazu Hisamatsu,* Susumu Okamoto,* Toshiro Sato,*
Katsuyoshi Matsuoka,* Kumiko Arai,* Takaaki Nakai,* Akira Hasegawa,* Nagamu Inoue,*
Noriaki Watanabe,[†] Kiyoko S. Akagawa,[‡] and Toshifumi Hibi[2]*

Disorders in enteric bacteria recognition by intestinal macrophages (Mϕ) are strongly correlated with the pathogenesis of chronic colitis; however the precise mechanisms remain unclear. The aim of the current study was to elucidate the roles of Mϕ in intestinal inflammation by using an IL-10-deficient (IL-10$^{-/-}$) mouse colitis model. GM-CSF-induced bone marrow-derived Mϕ (GM-Mϕ) and M-CSF-induced bone marrow-derived Mϕ (M-Mϕ) were generated from bone marrow CD11b$^+$ cells. M-Mϕ from IL-10$^{-/-}$ mice produced abnormally large amounts of IL-12 and IL-23 upon stimulation with heat-killed whole bacteria Ags, whereas M-Mϕ from wild-type (WT) mice produced large amounts of IL-10 but not IL-12 or IL-23. In contrast, IL-12 production by GM-Mϕ was not significantly different between WT and IL-10$^{-/-}$ mice. In ex vivo experiments, cytokine production ability of colonic lamina propria Mϕ (CLPMϕ) but not splenic Mϕ from WT mice was similar to that of M-Mϕ, and CLPMϕ but not splenic Mϕ from IL-10$^{-/-}$ mice also showed abnormal IL-12p70 hyperproduction upon stimulation with bacteria. Surprisingly, the abnormal IL-12p70 hyperproduction from M-Mϕ from IL-10$^{-/-}$ mice was improved by IL-10 supplementation during the differentiation process. These results suggest that CLPMϕ and M-Mϕ act as anti-inflammatory Mϕ and suppress excess inflammation induced by bacteria in WT mice. In IL-10$^{-/-}$ mice, however, such Mϕ subsets differentiated into an abnormal phenotype under an IL-10-deficient environment, and bacteria recognition by abnormally differentiated subsets of intestinal Mϕ may lead to Th1-dominant colitis via IL-12 and IL-23 hyperproduction. Our data provide new insights into the intestinal Mϕ to gut flora relationship in the development of colitis in IL-10$^{-/-}$ mice. *The Journal of Immunology*, 2005, 175: 6900–6908.

Macrophages (Mϕ),[3] the major population of tissue-resident mononuclear phagocytes, play key roles in bacterial recognition and elimination as well as in polarization of innate and adaptive immunities. Mϕ are activated by microbial pathogen-associated molecular patterns (PAMPs) through pattern-recognition receptors, such as TLRs (1, 2), and produce proinflammatory cytokines such as IL-12 and IL-23, thereby leading to development of Th1 immunity (3). Besides these classical antibacterial immune roles, it has recently become evident that Mϕ also play important roles in homeostasis maintenance, such as inflammation dampening, via production of anti-inflammatory cytokines such as IL-10 and TGF-β, debris scaveng-

ing, angiogenesis, and wound repair (4–6). IL-10 and IL-12 are two key players in these processes, usually acting in opposition, with IL-10 inhibiting IL-12 production. Therefore, loss of balance between IL-12 and IL-10 can lead to disproportionate pathology or immunosuppression.

Although precise etiologies of inflammatory bowel diseases (IBDs) including Crohn's disease and ulcerative colitis remain unclear, pathogenic roles of the gut flora in initiation and perpetuation of intestinal inflammation have been proposed (7). Recently, it has become evident that abnormal innate immune responses to bacteria are responsible for the pathogenesis of IBD. For instance, mutations in nucleotide-binding oligomerization domain (NOD)2 highly correlated with disease incidence in a substantial subgroup of patients with Crohn's disease (8, 9). NOD2 mutant Mϕ were reported to produce large amounts of IL-12 in response to stimulation with microbial components, compared with wild-type (WT) cells, and to promote Th1 immunity (10). Thus, disorders in bacterial recognition by Mϕ strongly correlate with pathogenesis of IBDs (11–13).

IL-10-deficient (IL-10$^{-/-}$) mice develop spontaneous chronic colitis and are widely used as a colitis animal model for human IBDs (14). IL-10$^{-/-}$ mice show Th1 polarized immunity upon exposure to bacteria, whereas germfree conditions prevent development of intestinal inflammation (15), and treatment with antibiotics attenuates intestinal inflammation (16, 17). These facts suggest that enteric bacteria play essential roles in onset and development of colitis in IL-10$^{-/-}$ mice, similar to human IBDs. Recently, the following pathogenic aspects of Mϕ in IL-10$^{-/-}$ mice have been reported: APC such as Mϕ and dendritic cells (DC), from IL-10$^{-/-}$ mice were potent activators of Th1 responses

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[3] Abbreviations used in this paper: Mϕ, macrophage; WT, wild type; DC, dendritic cell; IBD, inflammatory bowel disease; NOD, nucleotide-binding oligomerization domain; BM, bone marrow; M-Mϕ, M-CSF-induced BM-derived Mϕ; GM-Mϕ, GM-CSF-induced BM-derived Mϕ; PGN, peptidoglycan; MDP, muramyl-dipeptide; PAMP, pathogen-associated molecular pattern; MOI, multiplicity of infection; CBA, cytometric beads array; CLPMϕ, colonic lamina propria macrophage.

from naive or immune T cells (18, 19); Mϕ from IL-10$^{-/-}$ mice were hyperreactive to microbial components (20); and Mϕ depletion prevented chronic colitis in IL-10$^{-/-}$ mice (21). Based on these reports, Mϕ and DC are considered to play key roles in the pathogenesis of colitis in IL-10$^{-/-}$ mice, although mechanisms for bacterial recognition by APC that induces a Th1 bias and development of intestinal inflammation remain unclear. Previous studies suggested that IL-12 was crucial for development of colitis in IL-10$^{-/-}$ mice because mice deficient for both IL-10 and IL-12p40 showed no intestinal inflammation, and treatment with anti-IL-12p40 Abs markedly reduced intestinal inflammation (22, 23). Until now, however, how IL-10 deficiency affects IL-12 production from Mϕ in mice has not been thoroughly analyzed.

In the present study, we examined whether IL-10-deficient conditions affected differentiation and functions of bone marrow (BM)-derived Mϕ subsets and intestinal Mϕ, and investigated how bacteria recognition by Mϕ induced a Th1 polarization and intestinal inflammation in IL-10$^{-/-}$ mice. We found that M-CSF-induced BM-derived Mϕ (M-Mϕ) and intestinal Mϕ, but not GM-CSF-induced BM-derived Mϕ (GM-Mϕ) or splenic Mϕ from IL-10$^{-/-}$ mice showed abnormal hyperproduction of IL-12 and IL-23 upon stimulation with bacteria. More importantly, our results suggested that endogenous IL-10 played an essential role in differentiation of the anti-inflammatory Mϕ subset induced by M-CSF.

Materials and Methods

Reagents

Recombinant mouse GM-CSF, M-CSF, and IL-10 were purchased from R&D Systems. Gel filtration grade LPS (*Escherichia coli* O111:B4), muramyl-dipeptide (MDP), and *Staphylococcus aureus* peptidoglycan (PGN) were obtained from Sigma-Aldrich. Pam$_3$CSK$_4$ and *E. coli* ssDNA were obtained from InvivoGen.

Bacteria heat-killed Ags

A Gram-negative nonpathogenic strain of *E. coli* (25922; American Type Culture Collection (ATCC)) was cultured in Luria-Bertani medium, and a Gram-positive strain of *Enterococcus faecalis* (29212; ATCC) was cultured in brain-heart infusion medium. Bacteria were harvested and washed twice with ice-cold PBS. Then, bacterial suspensions were heated at 80°C for 30 min, washed, resuspended in PBS, and stored at −80°C. Complete killing was confirmed by 72 h incubation at 37°C on plate medium.

Mice

Specific pathogen-free WT C57BL/6J mice were purchased from Charles River Breeding Laboratories. WT and IL-10$^{-/-}$ (C57BL/6J background) mice were housed under specific pathogen-free conditions at the animal center of Kitasato Institute Hospital and Keio University (Tokyo, Japan). All experiments using mice were approved by and performed according to the guidelines of the animal committee of Keio University and Kitasato Institute Hospital.

Preparation of BM-derived Mϕ

BM cells were isolated from femora of 7- to 12-wk-old mice. After separation of BM mononuclear cells by gradient centrifugation, CD11b$^+$ cells were purified using a magnetic cell separation system (MACS; Miltenyi Biotec) with anti-mouse CD11b microbeads. To generate BM-derived GM-Mϕ and M-Mϕ, CD11b$^+$ cells (5 × 10^5 cells/ml) were cultured for 7 days with GM-CSF (20 ng/ml) and M-CSF (20 ng/ml), respectively. In some experiments, to determine effects of IL-10 during differentiation of M-Mϕ, BM CD11b$^+$ cells from IL-10$^{-/-}$ mice were cultured with M-CSF and various concentrations of exogenous IL-10. After differentiation, cells were washed three times with HBSS to remove residual IL-10.

Flow cytometry analysis

Day 7 BM-derived GM-Mϕ and M-Mϕ were harvested with EDTA and washed with ice-cold PBS. Then, cells were preincubated with 1 μg/ml mAb CD16/CD32 to block FcγR, and stained with mAbs CD11b, Gr-1, TLR4/MD2, or TLR2 (all from eBiosciences), mAbs CD80 or CD86 (both

from BD Pharmingen) or their isotype control Abs for 20 min at 4°C. After staining, cells were washed with PBS, stained with propidium iodide, and analyzed using a FACSCalibur (BD Pharmingen). The CellQuest software was used for data analysis.

Activation of BM-derived Mϕ by PAMPs

Day 7 BM-derived GM-Mϕ and M-Mϕ were harvested, plated on 96-well tissue culture plates (1 × 10^5 cells/well) in RPMI 1640 medium supplemented with 10% FBS, antibiotics, and 20 ng/ml GM-CSF or M-CSF, and incubated for 12–16 h. Before each experiment, Mϕ were washed three times with HBSS (Sigma-Aldrich) to completely remove secreted or supplemented cytokines from the supernatant, and were stimulated with either LPS (100 ng/ml), PGN (2 μg/ml), Pam$_3$CSK$_4$ (500 ng/ml), *E. coli* ssDNA (10 μg/ml), MDP (10 μg/ml), or heat-killed bacteria (multiplicity of infection (MOI) = 100) for 24 h. Culture supernatants were collected, passed through 0.22-μm pore size filters, and then stored at −80°C until the cytokine assay.

Isolation of colonic lamina propria Mϕ (CLPMϕ) and splenic Mϕ

Lamina propria mononuclear cells were isolated using a modified protocol as previously described (25). Briefly, mice were sacrificed, and colonic tissues were removed. Isolated colons were washed with HBSS, dissected into small pieces, and incubated in HBSS containing 2.5% FBS and 1 mM DTT (Sigma-Aldrich) to remove any mucus. Then, the pieces were incubated in HBSS containing 1 mM EDTA (Sigma-Aldrich) twice for 20 min each at 37°C, washed three times with HBSS, and incubated in HBSS containing 1 mM collagenase type IV (Sigma-Aldrich) for 2 h at 37°C. Digested tissues were filtered and washed twice with HBSS. Isolated cells were resuspended in 40% Percoll (Pharmacia Biotech), layered onto 75% Percoll, and centrifuged at 2000 rpm for 20 min. Cells were recovered from the interphase and washed with PBS. CLPMϕ and splenic Mϕ were purified by positive selection from lamina propria mononuclear cells or unfractionated splenocytes using MACS CD11b microbeads (Miltenyi Biotec) as previously described (24, 26).

Activation of Mϕ by whole bacteria Ags

BM-derived Mϕ, and isolated CLPMϕ and splenic Mϕ were plated on 96-well tissue culture plates (1 × 10^5 cells/well) in RPMI 1640 medium supplemented with 10% FBS and antibiotics, and stimulated by heat-killed bacterial Ags (MOI = 100) for 24 h at 37°C. Culture supernatants were collected, passed through a 0.22-μm pore size filter, and stored at −80°C until the cytokine assay.

Cytokine assay

A mouse inflammatory cytometric beads array (CBA) kit (BD Pharmingen) was used for cytokine measurements, according to the manufacturer's instructions. Samples were analyzed using a FACSCalibur (BD Pharmingen).

Quantitative RT-PCR

After 8 h of stimulation by bacterial Ags, total RNA was isolated from Mϕ using an RNeasy Mini kit (Qiagen). In some experiments, RNA was isolated from colonic tissues and spleen. cDNA was synthesized with OmniScript reverse transcriptase (Qiagen). For quantitative RT-PCR, TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays for murine IL-12p35, IL-12p40, IL-23p19, M-CSF, GM-CSF, and β-actin (Applied Biosystems) were used. PCR amplifications were conducted in a thermocycler DNA Engine (OPTICON2; MJ Research). Cycling conditions for PCR amplification were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

Statistical analysis

Statistical significance of differences between two groups was tested using a Student's *t* test. For comparison of more than two groups, ANOVA was used. If the ANOVA was significant, Dunnett's multiple comparison test or Scheffe's test were used as a post hoc test.

Results

GM-Mϕ and M-Mϕ derived from BM CD11b$^+$ cells from IL-10$^{-/-}$ mice do not differ significantly from those derived from WT mice in morphology and cell surface Ag expressions

When BM-derived CD11b$^+$ cells from WT mice were cultured in M-CSF or GM-CSF for 7 days, they showed morphological

changes characteristic of Mφ such as increases in size and adherence, and were stained with nonspecific esterase (data not shown). As shown in Fig. 1A, GM-CSF and M-CSF induced differentiation of BM CD11b⁺ cells into two distinct subsets of adherent Mφ, which corresponded to human GM-Mφ and M-Mφ (27). GM-Mφ derived from BM CD11b⁺ cells had a rounded morphology and possessed dendrites, similar to DCs. In contrast, M-Mφ derived

A

B

FIGURE 1. In vitro differentiated Mφ from WT and IL-10⁻/⁻ mice do not differ in morphology and surface marker expressions. A, BM CD11b⁺ cells from WT and IL-10⁻/⁻ mice were polarized into Mφ with GM-CSF or M-CSF for 7 days. B, Polarized Mφ from WT and IL-10⁻/⁻ mice (KO) were stained with the indicated mAbs and analyzed by flow cytometry. Profiles of specific Ab staining (shaded histograms) and staining with isotype controls (open histograms) are shown. Data shown are representative of five independent experiments.

from BM CD11b⁺ cells had an elongated spindle-like morphology. FACS analysis revealed that GM-Mφ expressed higher levels of MHC class II molecules and costimulatory molecules CD80 compared with M-Mφ (Fig. 1B and Table I). Expression of Gr-1 was also different between them; i.e., GM-Mφ but not M-Mφ expressed Gr-1 (Fig. 1B and Table I). However, CD11c, a DC marker, was not expressed on both Mφ (data not shown).

Next, we compared the two Mφ subsets derived from IL-10⁻/⁻ BM CD11b⁺ cells with those from WT BM CD11b⁺ cells. As shown in Fig. 1A, both GM-Mφ and M-Mφ from IL-10⁻/⁻ BM CD11b⁺ cells showed normal morphological characteristics. Flow cytometric analysis further revealed that these Mφ subsets from IL-10⁻/⁻ mice did not differ from those in WT mice in their cell surface Ag expressions (Fig. 1B and Table I). These results suggest that GM-Mφ and M-Mφ from IL-10⁻/⁻ mice are similar to those in WT mice, at least in terms of morphology and cell surface Ag expression.

BM-derived M-Mφ from WT mice show an anti-inflammatory phenotype in response to PAMPs and whole bacterial Ags

To determine the immunological responses of GM-Mφ and M-Mφ from WT mice to PAMPs stimulation, Mφ were stimulated with various kinds of PAMPs for 24 h, and production levels of IL-12p70 and IL-10 in culture supernatant were measured. As shown in Fig. 2A, none of the stimuli tested induced IL-12p70 production from both Mφ in WT mice. In contrast, the TLR4 ligand LPS, TLR2 ligands PGN and Pam₃CSK₄, and TLR9 ligand *E. coli* ssDNA induced IL-10 production by these Mφ, although the amounts produced were higher in M-Mφ compared with GM-Mφ. The NOD2 ligand MDP did not induce either IL-12p70 or IL-10 in either subset from WT mice.
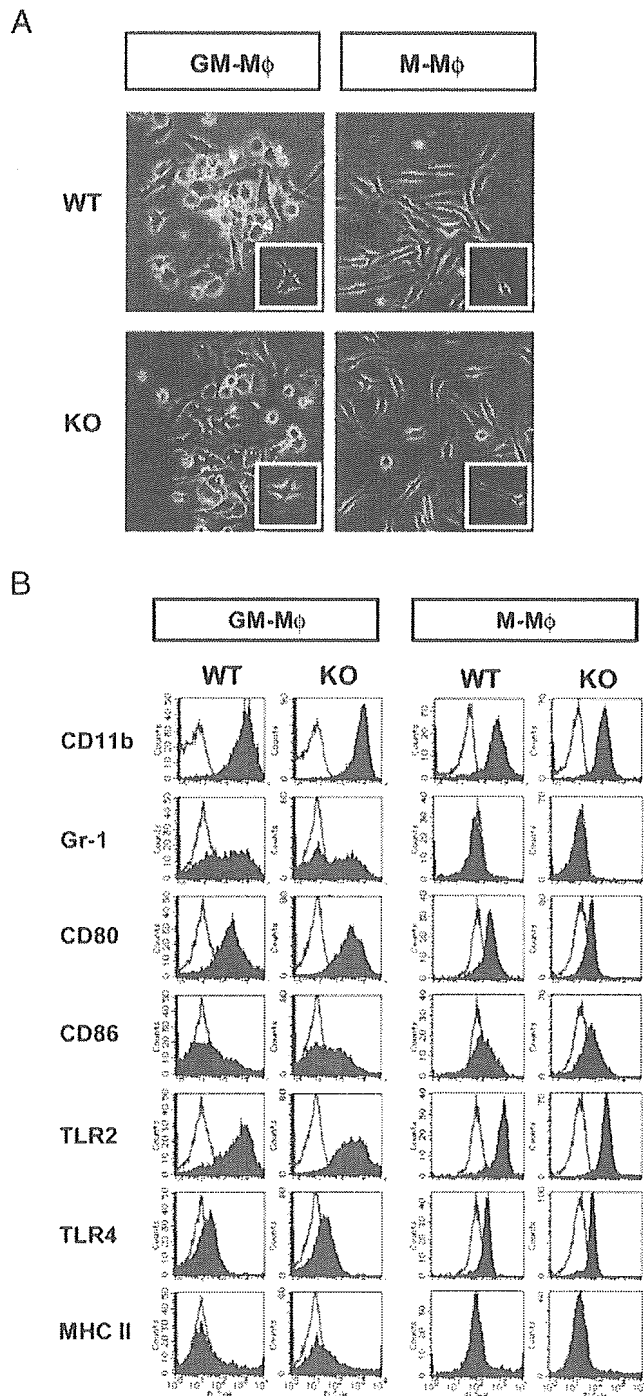
Next, we examined the effects of whole bacterial Ags on these Mφ. In contrast to stimulation with PAMPs, stimulation of GM-Mφ with heat-killed *E. coli* and *E. faecalis* induced IL-12p70 production (Fig. 2B). However, M-Mφ from WT mice did not produce IL-12p70, but did produce large amounts of IL-10 in response to the whole bacterial Ags (Fig. 2B). These results suggested that M-Mφ, but not GM-Mφ, in WT mice act as anti-inflammatory Mφ in the recognition of bacteria.

BM-derived M-Mφ but not GM-Mφ from IL-10⁻/⁻ mice reveal abnormal hyperproduction of IL-12 and IL-23 in response to whole bacterial Ags

We next examined the effects of PAMPs and whole bacteria Ags on GM-Mφ and M-Mφ from IL-10⁻/⁻ mice. In contrast to the results obtained from Mφ in WT mice, IL-10⁻/⁻ Mφ produced IL-12p70 by stimulation with LPS or Pam₃CSK4, although the amounts were very low, and no significant differences were observed between GM-Mφ and M-Mφ (Fig. 3A). The use of 10-fold higher concentrations of these PAMPs did not induce higher levels of IL-12p70 either (data not shown).

Upon whole bacteria stimulation, such as with heat-killed *E. coli* and *E. faecalis*, GM-Mφ from IL-10⁻/⁻ mice produced similar levels of IL-12p70 to WT GM-Mφ, although they lacked IL-10 production ability (Fig. 3B). Surprisingly, in contrast to WT M-Mφ, M-Mφ from IL-10⁻/⁻ mice produced significantly large amounts of IL-12p70 upon stimulation with whole bacterial Ags (Fig. 3B). In addition, a lower dose of the whole bacteria Ag (MOI = 10) also induced abnormally large IL-12p70 production (data not shown).

To further confirm this abnormal IL-12p70 hyperproduction by IL-10⁻/⁻ M-Mφ, gene transcriptions of IL-12p35, p40, and IL-23p19 were analyzed using real-time quantitative PCR. Results revealed that basal expressions of these genes before stimulation

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. 3C). 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. 3C).

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. 4A). Abnormal IL-12p70 hyperproduction by M-Mφ from IL-$10^{-/-}$ mice was completely suppressed by exogenous IL-10 in a dose-dependent manner (Fig. 4B). 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. 5A). 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. 5B).

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. 5C). 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. 6A). 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. 6A). 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. 6A). Similar results were obtained when CLPMφ from WT and IL-$10^{-/-}$ mice were stimulated with heat-killed *E. faecalis* (data not shown).
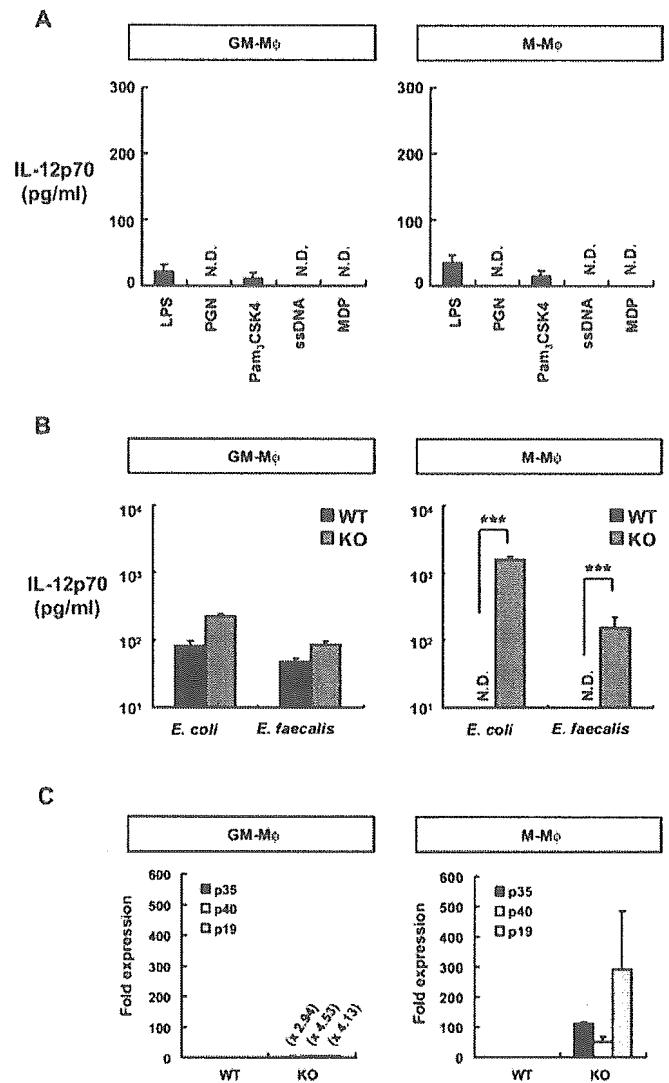
These results suggest that CLPMφ revealed a functional similarity to that of M-Mφ, that CLPMφ from WT mice act as anti-inflammatory Mφ via production of large amounts of IL-10, and that CLPMφ from IL-$10^{-/-}$ mice contribute to the development of Th1-dominant colitis via the abnormal hyperproduction of IL-12p70.
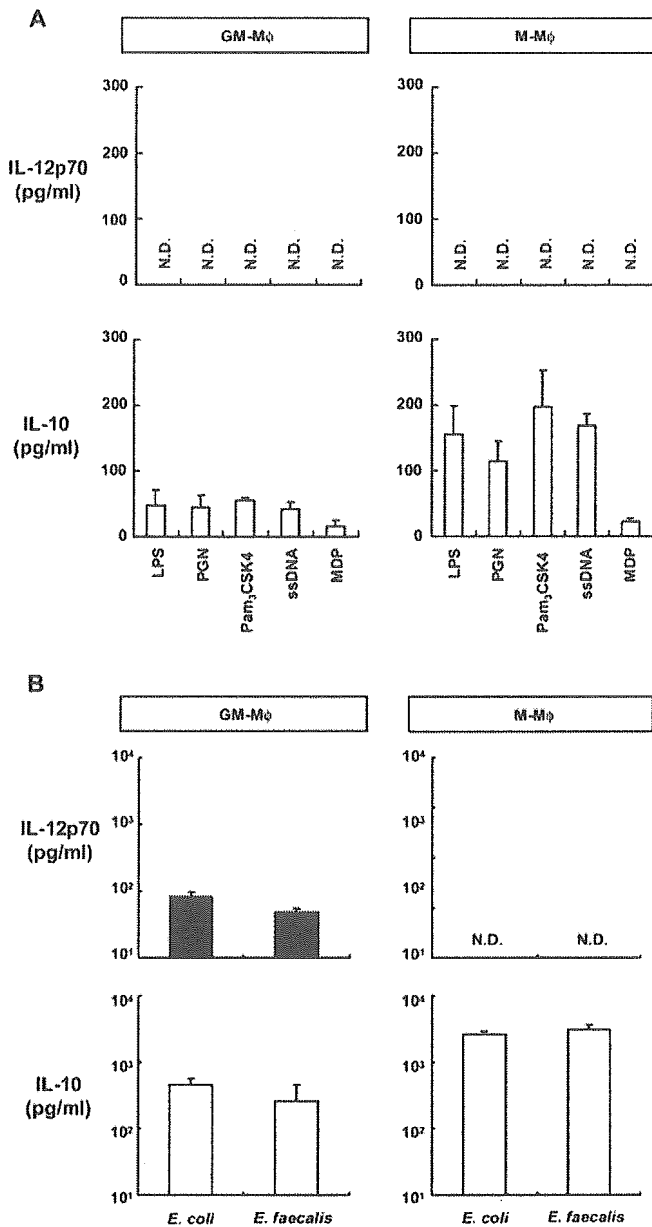
FIGURE 2. BM-derived M-Mϕ from WT mice reveal an anti-inflammatory phenotype in response to PAMPs and whole bacterial Ags. *A*, Polarized GM-Mϕ and M-Mϕ (1 × 10^5 cells) from WT mice were stimulated with LPS (100 ng/ml), PGN (2 μg/ml), Pam$_3$CSK$_4$ (500 ng/ml), *E. coli* ssDNA (10 μg/ml), or MDP (10 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$_3$CSK$_4$, 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$_3$CSK$_4$ (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$_3$CSK$_4$, 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

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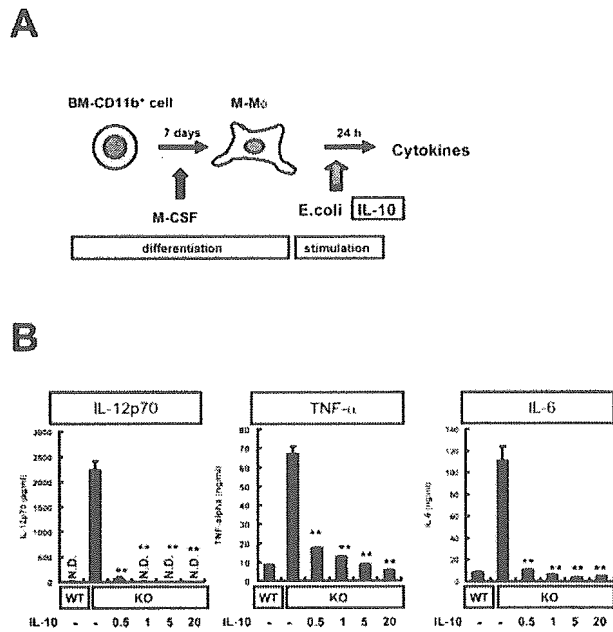
FIGURE 4. Exogenous IL-10 prevents the production of proinflammatory cytokines by M-Mϕ from IL-10$^{-/-}$ mice. *A*, Schema of the experiment. *B*, M-Mϕ (1 × 10^5 cells) from WT and IL-10$^{-/-}$ mice (KO) were stimulated with heat-killed *E. coli* (MOI = 100) for 24 h with or without various concentrations 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).

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 non-pathogenic 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

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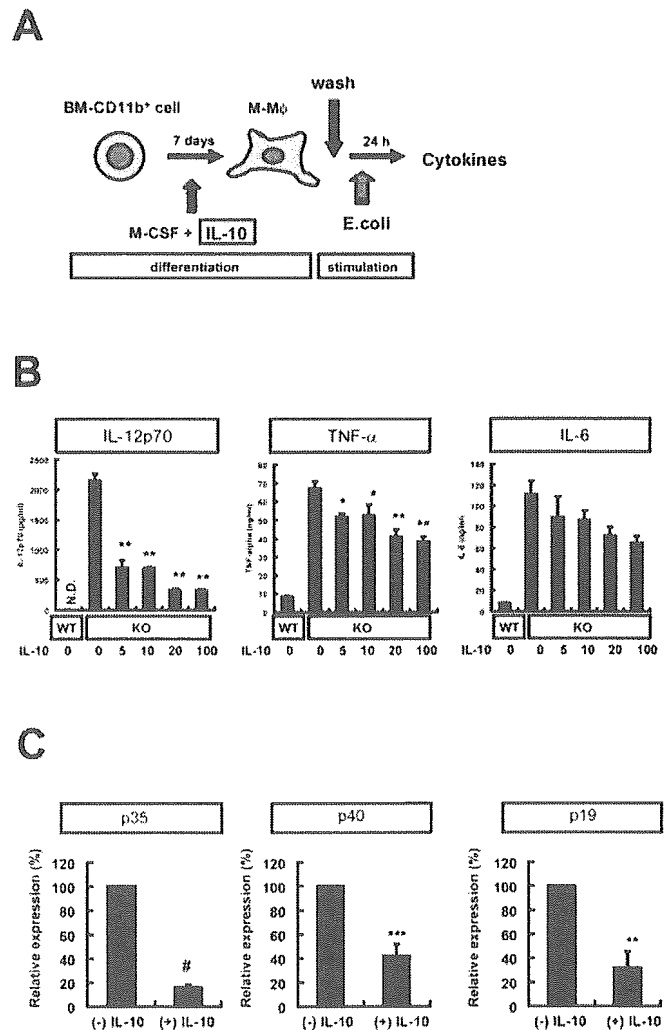
FIGURE 5. IL-10 supplementation during the differentiation process of M-Mϕ improves the abnormal IL-12 production upon stimulation with whole bacterial Ags. *A*, Schema of the experiment. *B*, BM CD11b$^+$ cells from IL-10$^{-/-}$ mice (KO) were differentiated with 20 ng/ml M-CSF and various concentrations 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).

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).

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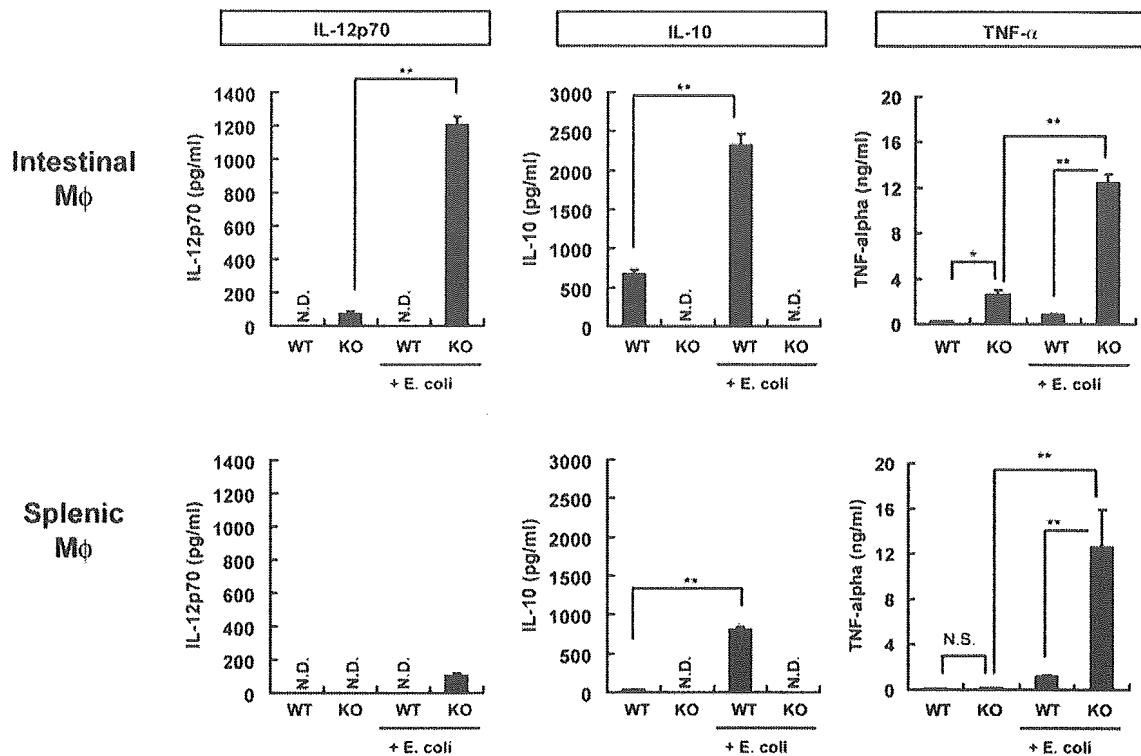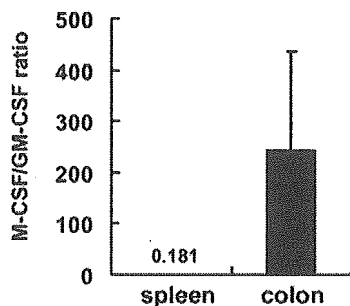
FIGURE 6. CLPMφ, but not splenic Mφ, from IL-10$^{-/-}$ mice produce a large amount of IL-12 upon stimulation with whole bacterial Ags. *A*, CLPMφ and splenic Mφ were isolated from WT and IL-10$^{-/-}$ mice (KO). Mφ were seeded at 1×10^5 cells/well and incubated with or without heat-killed *E. coli* (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.

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.

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

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

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

Up-regulated Smad5 Mediates Apoptosis of Gastric Epithelial Cells Induced by *Helicobacter pylori* Infection*

Tomokazu Nagasako, Toshiro Sugiyama‡, Takuji Mizushima, Yosuke Miura, Mototsugu Kato, and Masahiro Asaka

From the Department of Gastroenterology, Hokkaido University Graduate School of Medicine, Kita-15, Nishi-7, Kita-ku, Sapporo 060-8638, Japan

The gastric pathogen *Helicobacter pylori* activates epithelial cell signaling pathways, and its infection induces changes in the expression of several genes in infected human gastric tissues. Recent studies have indicated that the ability of *H. pylori* to regulate epithelial cell responses depends on the presence of an intact *cag* pathogenicity island (cagPAI). We investigated altered mRNA expression of gastric epithelial cells after infection with *H. pylori*, both cagPAI-positive and cagPAI-negative strains, by cDNA microarray, reverse transcription PCR, and Northern blot analysis. Our results indicated that cagPAI-positive *H. pylori* strains (ATCC 43504 and clinical isolated strains) significantly activated Smad5 mRNA expression of human gastric epithelial cells (AGS, KATOIII, MKN28, and MKN45). We further examined whether the up-regulated Smad5 was related to apoptosis of gastric epithelial cells induced by *H. pylori*. Smad5 RNA interference completely inhibited *H. pylori*-induced apoptosis. These results suggest that Smad5 is up-regulated in gastric epithelial cells through the presence of cagPAI of *H. pylori* and that Smad5 mediates apoptosis of gastric epithelial cells induced by *H. pylori* infection.

Helicobacter pylori is a human pathogen that infects the gastric mucosa and causes an inflammatory process leading to gastritis, gastric ulceration, duodenal ulceration, mucosa-associated lymphoid tissue lymphoma, and gastric cancer (1). The pathogenesis of gastroduodenal diseases caused by this bacterium is not well understood. Since the whole genome of *H. pylori* was sequenced in 1997, several putative virulence factors, including VacA (2), IceA, OipA (3), HrgA (4), lipopolysaccharide, and the neutrophil-activating protein (5), have been elucidated. The *cag* pathogenicity island (cagPAI),[1] a complex of genes coding ~30 proteins, has been reported to be a major virulence factor of *H. pylori*. The cagPAI is acquired by horizontal transfer and is found in about 50–70% of *H. pylori* isolates in Western countries and in more than 90% of *H. pylori* isolates in Asian countries, including Japan (6, 7). This lesion

codes for the type IV secretion machinery system forming a cylinder-like structure connected to epithelial cells (8). Many virulence gene products or other interactive proteins might be transferred into the host cells via this system. Peptic ulceration and gastric cancer occur in some people with *H. pylori* infection, but the majority remain asymptomatic. Although differences among the degrees of gastric mucosal damage caused by different strains should be an important factor for development of various clinical outcomes, these strain differences do not provide a complete explanation for individual differences in *H. pylori* infection-induced gastric mucosal injury. Therefore, it is presumed that host responses also play an important role in the outcome of *H. pylori* infection, interacting with virulence factors and environmental factors. Recent studies have shown that *H. pylori* induced various cellular responses, proliferation, apoptosis (9), cytoskeletal rearrangement (10), modification of intracellular signaling molecules (11), vacuolation (12), and cytokine secretion (13). In this study, we investigated the altered gene expression of host cells infected with cagPAI-positive or cagPAI-negative *H. pylori* strain and the association between the altered gene expression and the cellular responses.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Cell Lines—Biopsy specimens were obtained from Japanese patients in Hokkaido University Hospital and were cultured on *H. pylori*-selective agar plates (Eiken Chemical Co., Ltd., Tokyo, Japan) under microaerophilic conditions (5% O_2, 10% CO_2, 85% N2, at 37 °C; Aaero Pack Systems, Mitsubishi Gas Chemical, Osaka, Japan) for up to 5 days. Biopsies were obtained with informed consent from all patients under protocols approved by our ethics committee. The organisms were identified as *H. pylori* by spiral morphology and positive oxidase, urease, and catalase reactions. One colony on the agar was collected and cultured again under the same microaerophilic conditions in brain heart infusion broth (Nissui, Osaka, Japan) containing 5% (v/v) horse serum for up to 3 days. Aliquots were stored at −80 °C in 10% phosphate-buffered saline containing 20% (v/v) glycerol. After thawing of aliquots of the frozen culture, bacterial suspensions were cultured at 37 °C in brain heart infusion broth containing 10% fetal calf serum (Invitrogen) under microaerophilic conditions as described above on a gyratory shaker at 170 rpm for 24–36 h to the plateau phase. The human gastric cell lines AGS, KATOIII, MKN 28, MKN 45 were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and were maintained in a complete medium consisting of RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 0.2 mg of ampicillin/ml, and 100 μg of kanamycin/ml.

Co-culture of Epithelial Cells with H. pylori—Human gastric epithelial cell lines were cultured in RPMI 1640 containing 10% fetal calf serum without antibiotics and used at a final concentration of 5 × 10^5/ml. Bacterial suspensions were cultured at 37 °C in brain heart infusion broth containing 10% fetal calf serum under microaerophilic conditions as described above on a gyratory shaker at 170 rpm for 24–36 h to the plateau phase. The bacteria were then suspended in sterile phosphate-buffered saline. After centrifugation, the bacteria

[1] The abbreviations used are: cagPAI, *cag* pathogenicity island; siRNA,; short interference RNA; RT, reverse transcription; TGF, transforming growth factor; BMP, bone morphogenetic protein(s).

This paper is available on line at http://www.jbc.org

were resuspended at a final concentration of 1×10^7 colony-forming units/ml in RPMI 1640 supplemented with 10% fetal calf serum and used immediately. Gastric epithelial cells alone or cells with bacteria were cultured in tissue culture dishes (Falcon; Becton Dickinson) at 37 °C in a humidified incubator in an atmosphere of 95% air and 5% CO_2. The cells were washed with phosphate-buffered saline three times after 4, 8, 12, and 24 h. Total cellular RNA was extracted from the cells by using Isogen reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions, and the amount was measured by absorbance at 260 nm.

cDNA Microarray Procedure—Poly(A) RNA was isolated from total cellular RNA (100 μg) using an MagExtractor (Toyobo, Tsuruga, Japan) according to the manufacturer's instructions. Total cellular RNA was incubated with oligo(dT) magnetic beads (in the kit), and then nonspecific substance was removed by washing. 2 μg of mRNA was reverse transcribed into cDNA by reverse transcriptase, ReverTraAce (Toyobo), in the presence of a cDNA synthesis primer. Biotin-labeled probes were generated by binding of biotin-16-deoxyuridine triphosphate during synthesis of cDNA. The human cDNA expression filters, human cancer filters (Toyobo) were prehybridized at 62 °C for 30 min in 20 ml of PerfectHyb solution (Toyobo). After denaturalization, cDNA probes were hybridized to the filters overnight at 62 °C. The membranes were washed three times with solution 1 (2× SSC and 0.1% SDS) and three times with solution 2 (0.1× SSC and 0.1% SDS) for 5 min at 62 °C. Specific signals on the filters were detected by using a chemiluminescence detection kit, Imaging High (Toyobo), according to the manufacturer's instructions. CDP-Star was used as the chemiluminescent substrate. Images and quantitative data of gene expression levels were obtained using a Fluor-S Multiimager system (Nippon Bio-Rad Laboratories, Tokyo, Japan) and quantified into intensity of signals by using ImaGene (BioDiscovery, Inc., Los Angeles, CA).

Northern Blot Analysis—20 μg of total RNA was electrophoresed on a 1% agarose gel containing 6.5% formaldehyde and then transferred onto a nylon membrane. A Smad5 probe was made from human Smad5 cDNA that corresponded to its whole coding region. Each probe was labeled with biotin using Biotin-16-dUTP (Roche Diagnostics, Tokyo, Japan). A human β-actin probe labeled with biotin was used as a positive control. The membrane was hybridized with the labeled probe for 20 h at 62 °C in PerfectHyb (Toyobo). After hybridization, it was washed three times with 2× SSC with 0.1% SDS for 10 min and washed three times with 0.1× SSC with 0.1% SDS for 10 min at 62 °C. Positive bands were detected by using chemiluminescence detection kit (Imaging High; Toyobo), and CDP-Star was used as the chemiluminescent substrate according to the manufacturer's instructions.

mRNA Expression by RT-PCR—First strand cDNA templates were synthesized from 2 μg of total RNA using ReverTraAce and a random primer (Toyobo) according to the manufacturer's instructions. An aliquot (0.1 μl) of *Taq* DNA polymerase and deoxynucleoside triphosphates (Takara Shuzou Co., Ltd., Shiga, Japan) was mixed with 0.5 μl of a first strand cDNA sample and each primer. The primers used were Smad5F (5'-CAACACAGCCTTCTGGTTCA-3') and Smad5R (5'-TTGA-CAACAAACCCAAGCAG-3') for Smad5 amplification. PCR was performed using a thermal cycler (Takara Shuzou) under the following conditions: an initial denaturation for 5 min at 94 °C; 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C; and a final extension at 72 °C for 5 min with the number of cycles at which the band intensity increased linearly with the amount of mRNA used. The PCR product was then run on 1.5% agarose gel.

Analysis of Apoptosis—We used two methods to detect apoptosis of epithelial cells induced by *H. pylori* infection. After co-culture of AGS cells with *H. pylori* for 72 h, DNA was extracted from the control and treated cells using an apoptosis ladder detection kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Each DNA (5 μg) was electrophoresed in 2% agarose gels. The gels were photographed under ultraviolet light, and DNA ladder formation was observed. Next, we carried out quantitative analysis of apoptosis. AGS cells were cultured with *H. pylori* for 72 h in 96-well plates (2×10^4 cells/well). After centrifugation at 1500 rpm for 5 min, the supernatant was removed, and the pellets were frozen at −80 °C for 15 min. Then the terminal deoxynucleotidyl triphosphate-mediated deoxyuridine triphosphate nick end labeling assay was performed using a apoptosis screening kit (Wako Pure Chemical Industries, Ltd.) according to the manufacturer's instructions. The degree of apoptosis was evaluated numerically by measuring the absorbance (490 nm).

Interference of Smad5 mRNA—Two 29-mer DNA oligonucleotides (siRNA oligonucleotide templates) with 21 nucleotides encoding the siRNA and 8 nucleotides complementary to the T7 promoter primer were chemically synthesized, desalted, and purified by reverse phase
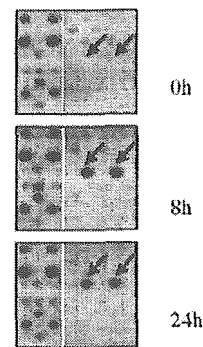

FIG. 1. *H. pylori* **up-regulated Smad5 expression in AGS cells.** cDNA microarray filters were hybridized with probes from AGS cells co-cultured with *H. pylori* (cagPAI-positive, ATCC 43504 strain) for 8 or 24 h or probes from AGS cells alone. The *arrows* indicate a differentially expressed Smad5 gene. The *left three lanes* indicate housekeeping genes.
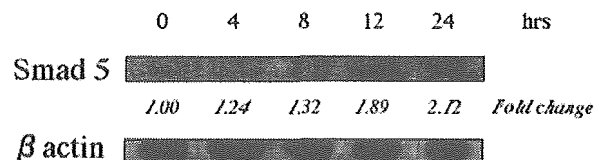

FIG. 2. *H. pylori* **up-regulated Smad5 expression in AGS cells by Northern blot analysis.** *H. pylori*-induced Smad5 mRNA expression of AGS cells was detected by Northern blot analysis. Total RNA was extracted from the cells co-cultured with *H. pylori* (ATCC43504 strain) for the indicated time intervals. *H. pylori* infection up-regulated Smad5 mRNA expression after 8 h of culture. The fold change of density was indicated. The β-actin probe was hybridized as a control.

high pressure liquid chromatography. These sequences were subjected to a BLAST search (NCBI data base) to ensure that only one gene was targeted. Two 21-mer oligonucleotides (sense, 5'-AATTACATCCTGC-CGGTGATA-3' and antisense, 5'-AATATCACCGGCAGGATGTAA-3') encoding Smad5 had no homology to those of Smad1, 2, 3, 4, and 8 in a BLAST search. The two siRNA oligonucleotide templates were hybridized to a T7 promotor primer and were extended by the Klenow DNA polymerase. The sense and antisense siRNA templates were transcribed by T7 RNA polymerase and were hybridized to create double-stranded siRNA using a Silencer siRNA construction kit (Ambion). The control and *H. pylori*-treated cells were grown in 96-well plates, and cationic lipid-mediated transient transfections were carried out with 50 ng of siRNA/well using GeneSilencer siRNA transfection reagent (Gene Therapy Systems, San Diego, CA). After incubation at 37 °C for 24 h, Northern blot analysis was performed to assess the effectiveness of RNA interference, and quantitative analysis of apoptosis was carried out as described above.

Statistics—The data are presented as the means ± S.D. The differences were examined by analysis of variance, and p values < 0.01 were considered significant.

RESULTS

Smad5 Up-regulation in Gastric Epithelial Cell Lines—We first examined changes in gastric cellular mRNA expression in response to co-culture with *H. pylori* (cagPAI-positive, ATCC 43504 strain) at 8 and 24 h by cDNA microarrays in AGS cells. Eleven housekeeping genes were used as internal controls to correct the mRNA abundance. Although the majority of genes indicated only small differences, the expression level of Smad5 mRNA increased dramatically (Fig. 1), with the relative fold changes in density to housekeeping genes being 0.4, 22.4, and 21.9 at 0, 8, and 24 h, respectively. The expression of the other Smad family (Smad1, 2, 3, 4, and 8) mRNA including R-Smads were increased 0.6–1.3-fold after 24 h co-culture and were not significant. Northern blot analysis was carried out to confirm the overexpression of Smad5 mRNA. Total RNA was extracted from AGS cells treated with *H. pylori* and untreated AGS cells at 4, 8, 12, and 24 h. Northern blot analysis showed that

FIG. 3. *H. pylori* up-regulated Smad5 expression in other human gastric epithelial cell lines and in native gastric mucosa. *a*, total RNA was extracted from human gastric epithelial cell lines (KATOIII cells, MKN28 cells, and MKN45 cells) co-cultured with live *H. pylori* for the indicated time intervals, and the expression of Smad5 mRNA was analyzed by RT-PCR using the specific primers. Smad5 mRNA expressions were up-regulated in all of the tested cells. The fold change of density was indicated. β-Actin was amplified as a control in parallel. *b*, total RNA was extracted from the five native gastric biopsy specimens from five patients infected with cagPAI-positive strains or the five native gastric biopsy specimens from five uninfected patients, and the expression of Smad5 mRNA was analyzed by RT-PCR using the specific primers. Smad5 mRNA were highly expressed in cagPAI-infected gastric mucosa. The fold change of density is indicated.
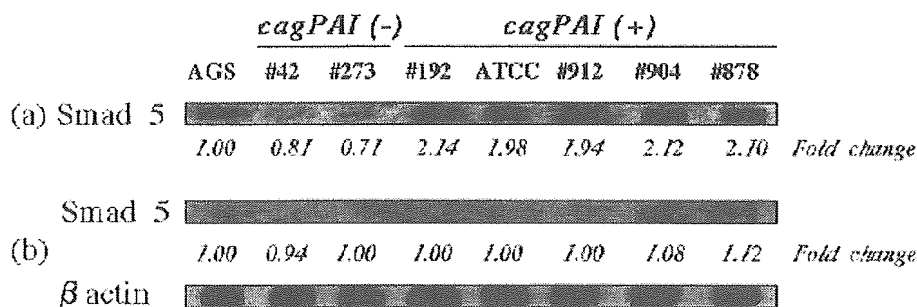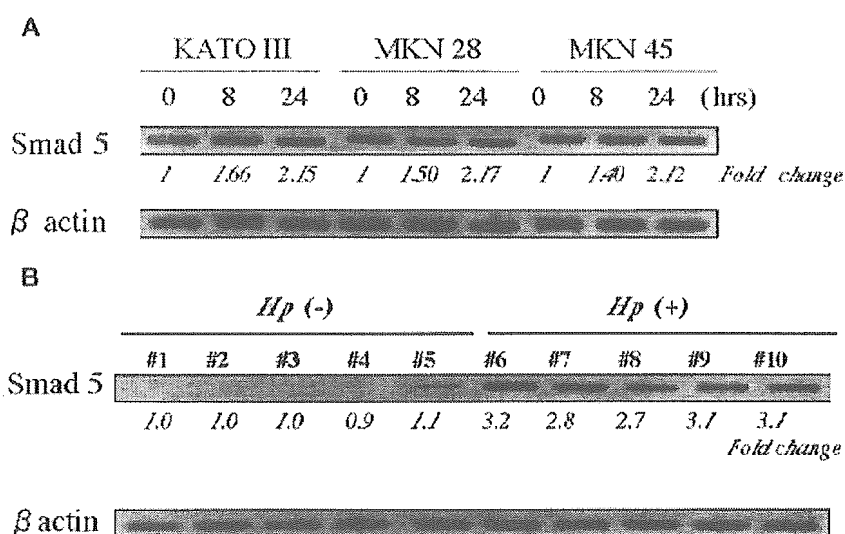

FIG. 4. **cagPAI-positive *H. pylori* up-regulated Smad5 expression of AGS cells by Northern blot analysis.** Smad5 mRNA expression of AGS cells after co-cultured with cagPAI-negative (strains 42 and 273) and cagPAI-positive (strains 192, ATCC 43504, 912, 904, and 878) *H. pylori* strains at 30 h. *a*, cagPAI-positive *H. pylori* strains induced up-regulation of Smad5 significantly, compared with cagPAI-negative strains. The fold change of density was indicated. *b*, after RNA interference, the up-regulated Smad5 mRNA expressions induced by *H. pylori* infection were suppressed in cagPAI-positive strains. The β-actin probe was hybridized as a control.

H. pylori infection up-regulated Smad5 mRNA expression of AGS cells after 4 h of co-culture (Fig. 2). We examined several other gastric epithelial cell lines (KATOIII, MKN28, and MKN45) to confirm Smad5 mRNA expression after co-culture with *H. pylori* by gene-specific RT-PCR. Smad5 mRNA was highly expressed after co-culture with *H. pylori* in all tested gastric epithelial cell lines (Fig. 3a).

Smad5 Up-regulation in Native Gastric Mucosa—We tested whether Smad5 mRNA was expressed in the 10 native gastric biopsy specimens by RT-PCR. Smad5 mRNA was highly expressed in the native gastric specimens from five patients infected with cagPAI-positive strains, however faintly expressed in the five uninfected gastric specimens (Fig. 3b).

Effects of cagPAI-positive and cagPAI-negative Strains—To assess the role of cagPAI in Smad5 mRNA expression, we tested cagPAI-positive strains (strains 192, ATCC 43504, 912, 904, and 878) and cagPAI-negative strains (strains 42 and 273) (14, 15) by Northern blot analysis. Strains 42 and 273 failed to up-regulate Smad5 mRNA expression, whereas cagPAI-positive strains clearly enhanced its expression (Fig. 4a).

Induction of Apoptosis—DNA fragmentation was induced in AGS cells 72 h after having been co-cultured with cagPAI-positive *H. pylori* strains (strains 192 and ATCC 43504), whereas no DNA fragmentation was observed with cagPAI-negative strains (strains 42 and 273) (Fig. 5a). Quantitative analysis of cellular apoptosis showed that cagPAI-positive *H. pylori* strains (strains 192, ATCC 43504, 912, 904, and 878) induced significantly greater levels of apoptosis in AGS cells than did cagPAI-negative strains and AGS cells alone (Fig. 5b).

The difference between the results obtained using cagPAI-positive strains and cagPAI-negative strains or AGS cells alone was statistically significant.

Interference of Smad5 Up-regulation and Apoptosis—The up-regulated Smad5 mRNA expression was inhibited by Smad5-specific RNA interference in all cagPAI-positive strain-infected AGS cells (Fig. 4b). With the suppression of Smad5 mRNA expression, the induction of apoptosis was completely inhibited in the quantitative apoptosis assay (Fig. 6).

DISCUSSION

The gastric pathogen *H. pylori* activates epithelial cell signaling pathways after infection. However, the exact signaling pathways are still unknown. The host immune response to *H. pylori* infection might be of importance with regard to the various clinical outcomes of infection by this organism. We now report that *H. pylori* can up-regulate the Smad5 expression of gastric epithelial cells and that the Smad5 up-regulation is involved in *H. pylori*-induced apoptosis of gastric epithelial cells. In addition, it was found that the presence of intact cagPAI is essential for Smad5-mediated apoptosis of epithelial cells.

We speculated that a paracrine or autocrine system of TGF-β and bone morphogenetic proteins (BMP) from infected *H. pylori* or AGS cells are involved in the up-regulation of Smad5 expression. In human gastric epithelial cells, AGS had TGF-β receptors (TGF-βRII) and BMP receptors, and up-regulation of Smad5 mRNA expression was observed after exogenous stimulation of TGF-β1 and BMP by Northern blot analysis (data
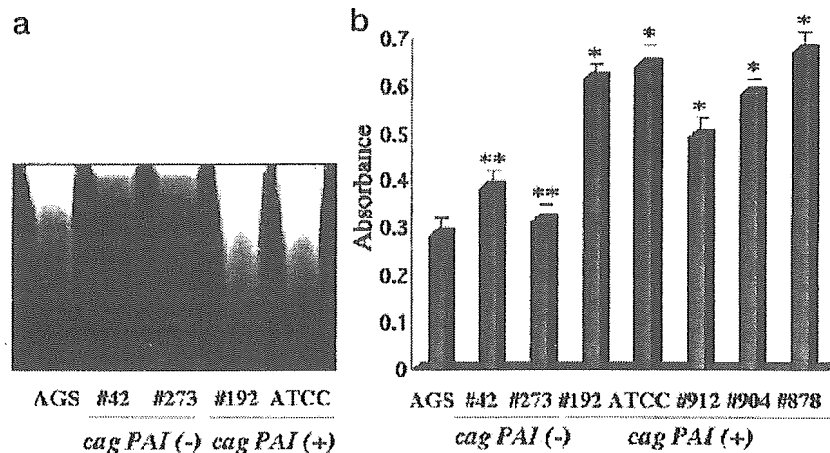
a

b

FIG. 5. *H. pylori*-induced apoptosis depended on the presence of cagPAI. *a*, DNA ladder formation. DNA fragmentation in AGS cells was induced by only cagPAI-positive *H. pylori* strains (strains 192 and ATCC 43504), and not by cagPAI-negative *H. pylori* strains (strains 42 and 273). *b*, quantitative analysis of apoptosis induced by cagPAI-positive and -negative *H. pylori* strains using a terminal deoxynucleotidyl triphosphate-mediated deoxyuridine triphosphate nick end labeling assay. Only cagPAI-positive *H. pylori* strains (strains 192, ATCC 43504, 912, 904, and 878) significantly induced apoptosis in AGS cells. The results are expressed as the absorbance (490 nm). The *bars* indicate the means of four independent experiments. *, $p < 0.01$ *versus* AGS alone. **, not significant *versus* AGS alone.

not shown). Although TGF-β1 and BMP in co-cultured supernatants from *H. pylori*-infected and uninfected AGS cells were measured by enzyme-linked immunosorbent assay, significant differences were not found (data not shown). Additionally, TGF-β1 mRNA and BMP mRNA were not up-regulated after co-culture with *H. pylori* in cDNA array experiments. Furthermore, because *H. pylori* itself did not possess TGF-β1 or BMP-like genes, which was examined by BLAST search (NCBI data base), it is unlikely that a paracrine or autocrine pathway of TGF-β or BMP from AGS infected with *H. pylori* or direct production of TGF-β or BMP from *H. pylori* is involved in up-regulation of Smad5 expression.

The cagPAI region encodes a novel *H. pylori* secretion system, type IV machinery (16), and this apparatus is essential for the induction of interleukin-8 via an NF-κB-dependent transcriptional process in human gastric cells (17, 18). It has recently been shown that CagA is injected from the attached *H. pylori* into host cells via the type IV machinery and that it forms a physical complex with SHP-2, the Src homology 2 domain-containing tyrosine phosphatase, in a phosphorylation-dependent manner and stimulates the phosphatase activity (11, 19). These findings suggest that protein or gene injection through the type IV machinery is a key mechanism for host-bacterial interaction induced by *H. pylori* infection. Consequently, it is not surprising that the transcriptional response of gastric epithelial cells is dependent on the presence of cagPAI. We therefore examined the Smad5 expression of AGS cells using cagPAI-positive and cagPAI-negative strains and that of the native gastric mucosa infected with *H. pylori*. Our results indicated that cagPAI-positive *H. pylori* strains were able to activate Smad5 mRNA expression and to induce apoptosis of the infected epithelial cells but that cagPAI-negative strains were not able to activate Smad5 mRNA expression or induce apoptosis. Although CagA is the only *H. pylori* protein known to translocate from the bacterium into the cell via the type IV secretion system, it can be assumed that transfer of unknown genes or gene products through the type IV machinery might be necessary for up-regulation of the Smad5 gene in host cells.

It has been reported that mutations in Smad4 played a significant role in the progression of colorectal tumors (20) and that a subset of families with juvenile polyposis had germ line mutations in the Smad4 gene and were at increased risk of
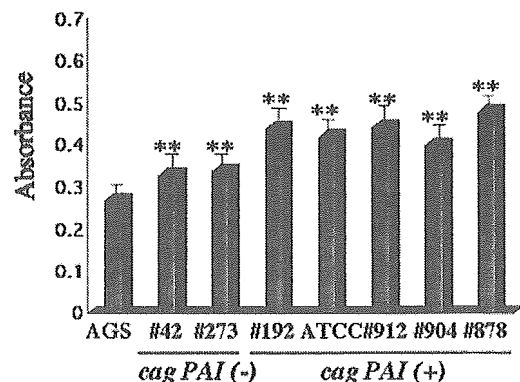

FIG. 6. *H. pylori*-induced apoptosis was reduced after specific suppression of Smad5 mRNA expression by RNA interference. Quantitative analysis of apoptosis induced by *H. pylori* strains after RNA interference. Interference with Smad5 mRNA expression suppressed the apoptosis of AGS cells co-cultured with cagPAI-positive strains (strains 192, ATCC 43504, 912, 904, and 878). The results are expressed as the absorbance (490 nm). The *bars* indicate the means of four independent experiments. **, not significant *versus* AGS alone.

developing gastrointestinal cancers (21). However, because there has been no report on Smad5 expression in gastrointestinal tract, the role of Smad5 in physiological or pathological status is not known.

Smad family proteins have molecular masses of about 42–65 kDa. Eight different Smads have been identified in mammals and can be classified into three subclasses, receptor-regulated Smads (R-Smads), common mediator Smads (Co-Smads), and inhibitory Smads (I-Smads) (22). Each member of the Smad family plays a different role in signaling pathways. R-Smads can be further subdivided into two subtypes, those phosphorylated after stimulation by TGF-β and BMP. Smad5 belongs to the latter group (23). Smad5 was isolated as dwarfin-C and was genetically implicated in TGF-β-like signaling pathways in *Drosophila* and *Caenorhabditis elegans* (24). Suzuki *et al.* (25) proposed that Smad5 directs the formation of the ventral mesoderm and epidermis in *Xenopus* embryos. In an antisense oligonucleotide study, Smad5 was shown to mediate the growth inhibitory effect in hematopoietic cells (26), and Yamamoto *et al.* (27) suggested that Smad5 inhibited myogenic differentiation. Furthermore, BMP actively mediated apoptosis in the embryonic limb (28), and BMP-2 also induced apoptosis in

human myeloma cell lines, probably via up-regulation of R-Smads (Smads1, 5, and 8) (29). Many studies have demonstrated that *H. pylori* induced apoptosis of gastric epithelial cells (30), suggesting that the up-regulated Smad5 mRNA expression might be involved in the apoptosis of gastric epithelial cells induced by *H. pylori* infection.

We also confirmed that only cagPAI-positive *H. pylori* strains were capable of inducing up-regulation of Smad5 mRNA as well as having apoptotic effects in human gastric cells. Although virulence factors, VacA, and lipopolysaccharide have been investigated as possible apoptosis-inducing factors (31), the precise intracellular signaling mechanism of apoptosis induced by *H. pylori* is still unknown. Our results indicated that Smad5 up-regulation might be related to the apotosis induced by cagPAI-positive *H. pylori* infection as one of the intracellular signaling molecules. We therefore compared the levels of *H. pylori*-induced apoptosis before and after suppression of Smad5 mRNA expression by RNA interference, and it was found that the induction of apoptosis was reduced to the background level after the interference. These observations suggest that Smad5 up-regulation is a key factor for *H. pylori*-induced apoptosis. In conclusion, *H. pylori* up-regulates Smad5 expression through the presence of cagPAI encoding type IV secretion machinery, and up-regulated Smad5 induces apoptotic responses in infected gastric epithelial cells.

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Review

Mucosal repair and growth factors: recombinant human hepatocyte growth factor as an innovative therapy for inflammatory bowel disease

Akio Ido[1], Masatsugu Numata[2], Mayumi Kodama[2], and Hirohito Tsubouchi[1,3]

[1] Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan
[2] Second department of Internal Medicine, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan
[3] Department of Digestive Disease and Life-style related Disease, Kagoshima University Graduate School of Medical and Dental Science, Kagoshima, Japan

The repair of intestinal mucosal injuries is a tightly regulated process involving epithelial restitution, cell proliferation and maturation, and the dedifferentiation of epithelial cells. Deeper injuries also require additional repair mechanisms, including inflammatory processes, angiogenesis, and extracellular-matrix deposition. Once intestinal mucosal injury occurs, numerous growth factors and cytokines, including hepatocyte growth factor (HGF), keratinocyte growth factor, endothelial growth factor, epidermal growth factor, transforming growth factor-β1, intestinal trefoil factor, interleukin (IL)-1, and IL-2, are induced in both the intestinal lumen and submucosa, and these factors cooperatively stimulate epithelial mucosal repair. HGF, a major agent promoting hepatocyte proliferation, also modulates intestinal epithelial cell proliferation and migration, leading to the acceleration of intestinal mucosal repair. Additionally, the proteolytic activation of HGF, which is mediated by HGF activator, is essential for the regeneration of injured intestinal mucosa. Recently, several studies have shown that the administration of recombinant human HGF or HGF gene therapy abrogates disease severity in several animal models of inflammatory bowel disease (IBD). Recombinant human HGF will soon be available for administration to patients with fulminant hepatic failure. Although additional preclinical biological studies are required, HGF has the potential to be an important new treatment modality promoting intestinal mucosal repair in patients with IBD.

Key words: mucosal repair, growth factor, HGF, HGF activator, inflammatory bowel disease
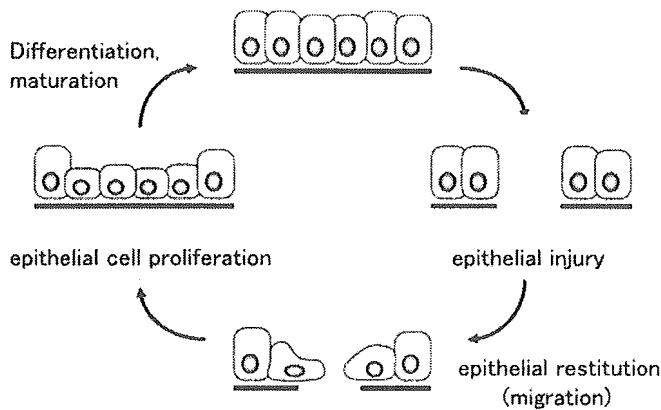
Introduction

Current treatments for inflammatory bowel disease (IBD) are predominantly anti-inflammatory and immune-modulating agents such as salazosulfapyridine, mesalazine, corticosteroids, azathioprine, 6-mercaptopurine, methotrexate, and cyclosporin.[1] Additionally, a chimeric mouse-human monoclonal antibody targeting tumor necrosis factor (TNF)-α has been developed, and this agent is extremely effective in controlling Crohn's disease.[2] Most IBD patients experience some benefit from current therapies, but the disease is often recurrent and intractable, frequently requiring surgical intervention. Recently, roles for several growth factors and cytokines in the repair of injured intestinal mucosa have been described,[3-7] and bone marrow-derived intestinal stem cells have been found within the injured intestinal mucosa.[8-10] This knowledge is expected to facilitate the generation of new therapeutic modalities that promote intestinal mucosal repair.

Hepatocyte growth factor (HGF), originally purified from the plasma of patients with fulminant hepatic failure, is the primary agent promoting hepatocyte proliferation.[11,12] HGF also functions as a pleiotropic factor, acting as a mitogen, morphogen, and motogen for multiple subsets of epithelial cells, including gastrointestinal epithelial cells.[11-16] In this article, we review recent work examining the involvement of growth factors in intestinal mucosal repair, as well as the use of recombinant human HGF as a new treatment for IBD that promotes the regeneration of injured intestinal mucosa.

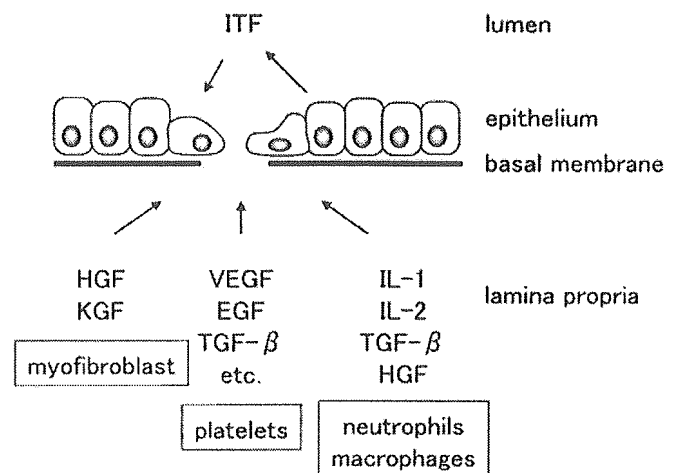## Intestinal mucosal repair and growth factors

The mucosal lining of the intestinal tract is comprised of a rapidly proliferating and continually renewing sheet of epithelial cells. Once the surface epithelium is injured, the continuity of the epithelial surface is

**Fig. 1.** Model of repair of intestinal epithelial cell injury

Fig. 2. Growth factors and cytokines are involved in the repair of intestinal mucosal injury. *HGF*, hepatocyte growth factor; *KGF*, keratinocyte growth factor; *VEGF*, vascular endothelial growth factor; *EGF*, epidermal growth factor; *TGF*, transforming growth factor; *IL*, interleukin; *ITF*, intestinal trefoil factor

reestablished by at least three distinct mechanisms (Fig. 1). First, epithelial cells bordering the zone of injury migrate into the wound to cover the denuded area. This process, termed epithelial restitution, does not require cell proliferation.[17-21] During restitution, epithelial cells adjacent to the injured surface undergo a striking change in cell shape and phenotype; the cells flatten and adopt a squamoid appearance, followed by the extension of pseudopodia-like structures. After closure of the wound, the cells reorganize their cytoskeleton and redifferentiate into mature enterocytes. Intestinal epithelial restitution occurs within minutes to hours after injury, both in vivo and in vitro. Secondly, epithelial cell proliferation is necessary to refill the decreased cell pool. Finally, the maturation and dedifferentiation of undifferentiated epithelial cells is required for the maintenance of mucosal epithelial function. When an epithelial defect is large, cell proliferation is critical for the restoration of normal mucosal architecture. Conversely, deeper lesions or penetrating injuries require additional repair mechanisms, including inflammation, angiogenesis, and the deposition of extracellular-matrix components, leading to the formation of granulation tissue.

Recently, numerous growth factors and cytokines, induced in both the lumen and the submucosa following mucosal injuries, have been identified (Fig. 2). These factors control a wide array of biological activities, including stimulating cell proliferation and migration, modulating cell differentiation, accelerating angiogenesis and extracellular-matrix remodeling, and promoting epithelial mucosal repair.[3-7] Following intestinal mucosal injury, myofibroblasts beneath the epithelial defect secrete HGF and keratinocyte growth factor (KGF). These growth factors stimulate both the migration and proliferation of epithelial cells.[22] HGF is also released from an intracellular pool by polymorphonuclear neutrophils.[23] Additionally, proinflammatory cytokines, including interleukin (IL)-1 and IL-2, are se-

creted by macrophages and mononuclear cells infiltrating the submucosa, and platelets also release several growth factors, such as vascular endothelial growth factor,[24] epidermal growth factor (EGF),[25] transforming growth factor (TGF)-β1,[26] and insulin-like growth factor.[27] These growth factors and cytokines interact predominantly with receptors on the basolateral membrane of epithelial cells. Conversely, intestinal trefoil factor (ITF) is secreted into the intestinal lumen by goblet cells following mucosal injury, and it acts primarily at the apical surface. ITF largely remains in the lumen, maintaining contact with the apical membrane via interactions with mucus. ITF promotes epithelial cell migration, and mice lacking the *ITF* gene are extraordinarily sensitive to mucosal injury and fail to undergo any epithelial repair.[28]

Matsuura et al.[29] have recently reported that the rectal administration of recombinant basic fibroblast growth factor (bFGF) ameliorated experimental colitis in a dose-dependent manner. bFGF stimulates epithelial restitution, as well as cell proliferation, through a TGF-β-dependent pathway in vitro,[30,31] and it also increases TGF-β production in colonic epithelial and fibroblast cell lines.[29]

Role of HGF in intestinal mucosal repair

HGF is a heparin-binding glycoprotein acting as a mitogen, morphogen, motogen, and/or angiogenic factor for a variety of cells through MET receptor tyrosine kinase.[32,33] Mesenchymal cells secrete HGF as an inactive single-chain precursor with an approximate mo-
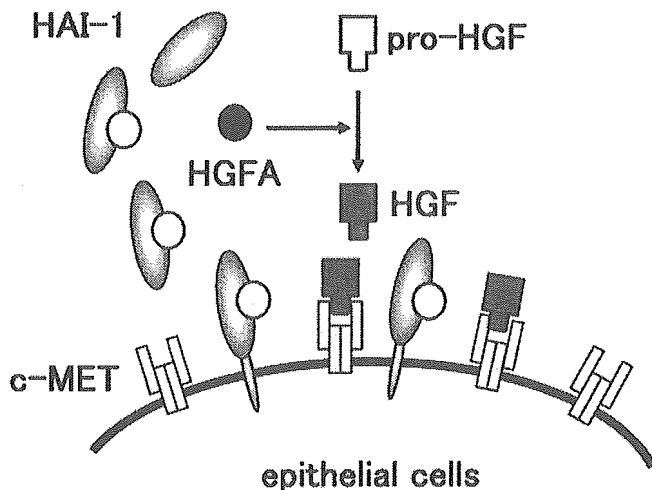
Fig. 3. HGF-associated molecules involved in the activation of HGF in injured mucosa. *HGFA*, HGF activator; *HAI-1*, HGF activator inhibitor type 1

lecular weight of 90 kilodaltons. Biologically active HGF is generated by the proteolytic cleavage of the inactive single-chain molecule, between Arg494 and Val495, to yield the active two-chain heterodimeric form.[34] Only active HGF can bind MET and transmit signals in target cells, including epithelial and endothelial cells. HGF activator (HGFA) is a serum serine proteinase that efficiently cleaves and activates HGF.[35,36] HGFA is primarily produced in the liver and is secreted into the plasma as an inactive proform.[36-38] Inactive pro-HGFA is activated by thrombin in injured tissues following cleavage between Arg407 and Ile408, leading to a two-chain active form.[38] Thus, HGF is exclusively activated by active HGFA in injured tissues that require the repair-promoting activity of HGF.[39-41] Recently, HGFA inhibitor (HAI)-1 was identified.[42] HAI-1 is a cell-surface binding protein that regulates HGFA activity in the pericellular microenvironment (Fig. 3).[43,44]

HGF, originally identified as a potent mitogen for hepatocytes, exerts a variety of activities on multiple subsets of epithelial cells, including gastrointestinal epithelial cells.[15,16,45] HGF functions as a positive growth regulator and accelerates remodeling after epithelial injury.[46-48] Indeed, HGF expression was upregulated in inflamed ulcerative colitis,[49,50] and plasma HGF levels were increased in animal models of acute colitis.[51,52] Additionally, both *c-met* and HGF are expressed in intestinal mucosal epithelial cells during recovery from experimental mucositis.[53] These findings suggest that HGF plays an important role in intestinal mucosal wound healing. Recently, Itoh et al.[54] reported that HGFA-deficient mice exhibited impaired intestinal mucosal regeneration following injury. In these mice, HGF activation was impaired in the injured mucosa,

and the injured colonic mucosa was not sufficiently covered by regenerated epithelium. These results indicate that the proteolytic activation of HGF, which is exclusively mediated by HGFA, is required for the repair of injured intestinal mucosa.

Therapeutic effect of HGF on experimental colitis

Recently, HGF was found to promote colonic mucosal repair in animal models of experimental colitis. Dextran sulfate sodium (DSS)-induced colitis is a well-established experimental model that mimics many of the signs and symptoms of human ulcerative colitis, including diarrhea, bloody feces, weight loss, mucosal ulceration, and shortening of the large intestine.[55,56] We reported that the continuous intraperitoneal delivery of recombinant human HGF reduced colitis-associated weight loss and large-intestinal shortening, and improved colonic erosions in rats with DSS-induced colitis (Fig. 4).[57] Compared with mock-treated rats, animals treated with recombinant human HGF exhibited enhanced epithelial regeneration and cellular proliferation. The rectal administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS) to immunocompetent mice results in transmural intestinal leukocyte infiltration accompanied by severe diarrhea and weight loss, resembling some characteristics of Crohn's disease.[58,59] Using this model, we further showed that the intravenous administration of recombinant human HGF to these rats with TNBS-induced colitis once a day for 5 days led to a significant reduction in colonic ulcer coverage and large-intestinal shortening (Fig. 5).[60] Repeated intravenous injections of recombinant human HGF stimulated the proliferation of epithelial cells and reduced the inflammatory cell infiltrate and the corresponding increase in TNF-α levels in the TNBS-inflamed colonic tissues. The effect of exogenous HGF on the immune and inflammatory cells in the submucosa remains obscure. However, it is possible that the HGF-induced enhancement of mucosal repair allows for a more rapid recovery of epithelial barrier function, and, therefore, the observed reduction of inflammation in HGF-treated rats may result from a decreased exposure to luminal stimuli, rather than from the direct influence of HGF on immune and inflammatory cells. The suppression of proinflammatory cytokines was also observed in several in vivo experiments using HGF gene therapy. Repeated transduction of naked HGF cDNA into skeletal muscle ameliorated enteropathy in murine models of acute graft-versus-host disease and TNBS-induced colitis.[61,62] In these experiments, the intestinal expression of T-helper 1 cytokines, including IL-12 and IL-1β, interferon-γ, and TNF-α, was also suppressed. Furthermore, the continuous intravenous infusion of recombi-