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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

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‡*

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), the majority 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 scavenging, 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.
from naïve 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 APCs which 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 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), muramyldipeptide (MDP), and Staphylococcus aureus peptidoglycan (PGN) were obtained from Sigma-Aldrich. Pam3CSK4 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 killing was confirmed by 72 h incubation at 37°C on plate medium. Bacteria were harvested and washed twice with ice-cold PBS. Then, bacterial suspensions were heated at 80°C for 10 min, washed with ice-cold PBS. Then, bacteria 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 eBioscience), 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 × 105 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), Pam3CSK4 (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, disected 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 unfraccionated splenocytes using MACS CD11b microbeads (Miltenyi Biotech) 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 × 105 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 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 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 bacterial 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

**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.
Therefore, we examined the role of IL-10 in differentiation of M-M to inhibit IL-12p70 production in M-M; these cytokines but rather produce anti-inflammatory cytokine IL-10. Moreover, consistent with IL-12p70 results, exogenous IL-10 was unable to induce large amounts of IL-12p70 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.

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 were 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).

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.

Table I. Expression of surface Ags on BM-derived different subsets of macrophage

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<td>GM-Mφ</td>
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<tr>
<td>CD11b</td>
<td>1174.2 ± 155.5*</td>
<td>509.5 ± 58.7</td>
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<td>Gr-1</td>
<td>93.1 ± 3.4*</td>
<td>0.0 ± 0.0</td>
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<tr>
<td>CD80</td>
<td>290.4 ± 34.2*</td>
<td>73.4 ± 27.5</td>
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<tr>
<td>CD86</td>
<td>14.5 ± 1.6</td>
<td>36.2 ± 8.1</td>
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<tr>
<td>TLR2</td>
<td>348.6 ± 18.6</td>
<td>342.4 ± 118.8</td>
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<tr>
<td>TLR4/MD2</td>
<td>7.6 ± 2.1</td>
<td>18.4 ± 0.8</td>
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<td>MHCI-II</td>
<td>20.6 ± 3.7*</td>
<td>1.7 ± 0.4</td>
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<tr>
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<th>GM-Mφ</th>
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<td></td>
<td>1229.7 ± 15.5*</td>
<td>388.2 ± 58.5</td>
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<td>63.7 ± 7.6*</td>
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<td>383.0 ± 4.1*</td>
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<td>26.7 ± 0.9</td>
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<td>386.0 ± 48.8</td>
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<td>8.7 ± 4.3</td>
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<td>24.4 ± 5.9*</td>
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* 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).
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 M/M-H9278 as described. M-CSF to GM-CSF expression level ratios in murine colonic tissues were dramatically higher than in spleen (Fig. 6B). These results demonstrate that M-CSF rich environment in colonic tissues may contribute the differentiation of intestinal M/M-H9278 into anti-inflammatory M-M/H9278 phenotype.

**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), Pam3CSK_{1} (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, Pam3CSK_{1}, 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.

**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
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 are 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 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.

**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 five independent experiments. N.D., Not detected. *p < 0.05; **p < 0.01 compared with IL-10−/− Mφ 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).
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.

References
ABNORMALLY DIFFERENTIATED INTESTINAL MΦ IN COLITIS


