
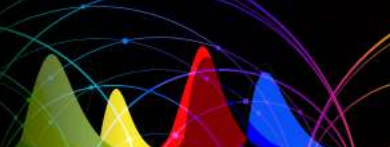


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Monocyte Chemoattractant Protein-1 Contributes to Gut Homeostasis and Intestinal Inflammation by Composition of IL-10–Producing Regulatory Macrophage Subset

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J Immunol 2010; 184:2671-2676; Prepublished online 27 January 2010;
 doi: 10.4049/jimmunol.0804012
<http://www.jimmunol.org/content/184/5/2671>

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The Journal of Immunology is published twice each month by
 The American Association of Immunologists, Inc.,
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 Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Monocyte Chemoattractant Protein-1 Contributes to Gut Homeostasis and Intestinal Inflammation by Composition of IL-10–Producing Regulatory Macrophage Subset

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Lamina propria macrophages (LPM ϕ s) spontaneously produce large amounts of anti-inflammatory IL-10 and play a central role in regulation of immune responses against commensal bacteria. MCP-1 is a chemokine that plays an important role in recruitment of monocytes and macrophages to inflamed tissues. We demonstrated that, in addition to IL-10, LPM ϕ s produced large amounts of MCP-1, even in a steady state. MCP-1 deficiency caused impaired IL-10 production by LPM ϕ s and led to exacerbation of dextran sulfate sodium-induced acute colitis. As an explanation of this impaired IL-10 production by LPM ϕ s, we found that LPM ϕ s could be separated into two subsets with distinct side-scattered properties, namely LPM ϕ 1 (CD11b⁺F4/80⁺CD11c[~]SSC^{hi}) and LPM ϕ 2 (CD11b⁺F4/80⁺CD11c[~]SSC^{lo}). Unlike LPM ϕ 1, the LPM ϕ 2 subset migrated in response to MCP-1 and produced a larger amount of IL-10 in response to commensal bacteria. LPM ϕ s isolated from MCP-1–deficient mice produced less IL-10 as a consequence of the lack of the MCP-1–dependent LPM ϕ 2 population. This imbalanced composition in LPM ϕ population may be involved in the susceptibility to DSS-induced colitis in MCP-1–deficient mice. Our results suggest that endogenous MCP-1 contributes to the composition of resident LPM ϕ subsets in the intestine. Moreover, MCP-1–dependent LPM ϕ 2 subset may play an important role in maintenance of gut homeostasis in the steady state, and in the termination of excess inflammatory responses in the intestine, by producing IL-10. *The Journal of Immunology*, 2010, 184: 2671–2676.

Macrophages are the major population of tissue-resident mononuclear phagocytes, and they play a key role in bacterial recognition and elimination, as well as in the polarization of innate and adaptive immunity. Besides these classical antibacterial immune roles, it has recently become evident that macrophages also play an important role in maintenance of homeostasis—for example, inflammation dampening via the production of anti-inflammatory cytokines such as IL-10 and TGF- β , debris scavenging, angiogenesis, and wound repair (1–3). Furthermore, recent studies have shown that M1 and M2 macrophages are functionally polarized in response to microorganisms and host mediators. M1 macrophage is characterized by producing proinflammatory cytokines such as TNF- α , IL-12, and IL-23, whereas M2 macrophage is characterized as producing IL-10 (2). Because the intestinal mucosa is always exposed to numerous commensal bacteria, it is thought that the gut may possess regulatory mechanisms that prevent excessive inflammatory responses

against commensal bacteria. It has been reported previously that intestinal macrophages do not express innate response receptors (4, 5), and although these cells retain their phagocytic and bactericidal functions, they do not produce proinflammatory cytokines in response to several inflammatory stimuli, including microbial components (6, 7). In contrast to splenic macrophages, recent studies have revealed that murine intestinal macrophages express several anti-inflammatory molecules, including IL-10, and induce differentiation of Foxp3⁺ T regulatory cells (Tregs) that are dependent on IL-10 and retinoic acid. Moreover, such intestinal macrophages suppress the intestinal dendritic cell-derived Th1 and Th17 immunity, which is dependent on or independent of Treg induction (7, 8). Thus, recent studies have suggested that macrophages located in the intestinal mucosa play important roles in the maintenance of intestinal homeostasis by protecting the host from foreign pathogens and negatively regulating excess immune responses to commensals (9). The regulation and composition of macrophages in the lamina propria (LP) is still not fully understood.

MCP-1 is a CC chemokine, which plays an important role in the recruitment of monocytes and macrophages from the bloodstream to inflamed tissue. MCP-1 is postulated to play a pivotal role in the pathogenesis of a variety of diseases that are characterized by mononuclear cell infiltration. In fact, it has been reported previously that MCP-1 deficiency reduces atherosclerosis. Atherosclerosis is reduced in low-density lipoprotein receptor-deficient mice, which are known as a model of atherosclerosis under MCP-1–deficient conditions. In these mice, infiltration of macrophages into the aortic wall is decreased (10, 11). In a mouse model of experimental autoimmune encephalomyelitis, MCP-1 deficiency leads to decreased local macrophage recruitment, which results in a diminished Th1 immune response (12). Thus, it is considered that locally infiltrated macrophages play a crucial role in the

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Received for publication December 1, 2008. Accepted for publication December 9, 2009.

This work was supported in part by grants-in-aid from the Japanese Ministry of Education, Culture and Science, the Japanese Ministry of Health, Labor and Welfare, Keio University, and Keio Medical Foundation, Tokyo, Japan.

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Abbreviations used in this paper: BM, bone marrow; BMM ϕ , bone marrow-derived macrophage; CBA, cytometric beads array; DSS, dextran sulfate sodium; FSC, forward scatter; KO, knockout; LP, lamina propria; LPM ϕ , lamina propria macrophage; LPT cell, LP CD3⁺ T cell; MCP-1^{–/–}, MCP-1–deficient; MOI, multiplicity of infection; SPM ϕ , splenic macrophage; SSC, side scatter; Treg, T regulatory cell; WT, wild-type.

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pathogenesis of several mouse disease models. Consistent with those observations, in intestinal inflammation it has been reported that MCP-1 expression is elevated at the inflamed sites in inflammatory bowel diseases (13). However, it remains unknown whether recruited intestinal macrophages have a proinflammatory phenotype that contributes to worsening of inflammation or an immunosuppressive phenotype that terminates inflammation.

We hypothesized that intestinal macrophages recruited by MCP-1 play a role in reducing inflammation via the production of anti-inflammatory cytokines, such as IL-10, because intestinal macrophages produce large amounts of IL-10. In the current study, we demonstrate that intestinal macrophages recruited by MCP-1 produce IL-10 and play an important role in amelioration of intestinal inflammation in murine experimental colitis models.

Materials and Methods

Mice

Breeding pairs of MCP-1-deficient (MCP-1^{-/-}) mice on a C57BL/6 background (14) were provided by B. Rollins (Dana-Farber Cancer Institute, Boston, MA). Appropriate wild-type (WT) C57BL/6 mice were purchased from Charles River Breeding Laboratories (Boston, MA). All animals were kept and bred at the animal facilities of Keio University (Tokyo, Japan); only 8–20-wk-old mice were used. All experiments using mice were approved by and performed according to the guidelines of the animal committee of Keio University.

Isolation of lamina propria macrophages

LP mononuclear cells were isolated using a modified protocol as described previously (15). Mice were sacrificed, and colonic tissues were removed. Isolated colons were washed with RPMI 1640, dissected into small pieces, and incubated in RPMI 1640 containing 2.5% FBS and 1 mM DTT (Sigma-Aldrich, St. Louis, MO) to remove any mucus. The pieces were incubated twice in RPMI 1640 containing 1 mM EDTA (Sigma-Aldrich) for 20 min each at 37°C, washed three times with RPMI 1640, and incubated in RPMI 1640 containing 1 mM collagenase type IV (Sigma-Aldrich) for 2 h at 37°C. Digested tissues were filtered and washed twice with RPMI 1640. Isolated cells were resuspended in 40% Percoll (Pharmacia Biotech, Uppsala, Sweden), layered onto 75% Percoll, and centrifuged at 2000 rpm for 20 min. Cells were recovered from the interphase and washed with PBS. Lamina propria macrophages (LPMφs) were purified by positive selection from LP mononuclear cells using a magnetic cell separation system (MACS; Miltenyi Biotec, Auburn, CA) with anti-mouse CD11b microbeads, as described previously (16, 17).

Cell sorting

Magnetically isolated LP CD11b⁺ cells were stained with FITC-conjugated CD11c mAb and PE-conjugated CD11b mAb (BD Pharmingen, San Diego, CA) after Fc receptor blockade. CD11b⁺CD11c^{SSC^{hi}} and CD11b⁺CD11c^{SSC^{lo}} LPMφ subsets were sorted with Epics Altra with the HyperSort cell sorting system (Beckman Coulter, Fullerton, CA), respectively. The purity of each LPMφ subset sorted was >90% by postsorting analysis.

Preparation of bone marrow-derived macrophages

Bone marrow (BM) cells were isolated from femora of 8–20-wk-old mice. After separation of BM mononuclear cells by gradient centrifugation, CD11b⁺ cells were purified using MACS. To generate bone marrow-derived macrophages (BMMφs), CD11b⁺ cells (5 × 10⁵ cells/ml) were cultured for 7 d with M-CSF (20 ng/ml; R&D Systems, Minneapolis, MN).

Bacterial heat-killed Ags

Enterococcus faecalis (29212; ATCC) was cultured in brain–heart infusion medium. Bacteria were harvested and washed twice with ice-cold PBS. 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.

Activation of LPMφs and BMMφs by whole bacterial antigens

Isolated LPMφs and BMMφs 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 *E. faecalis* (multiplicity of

infection [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 system (BD Pharmingen).

Flow cytometric analysis

Isolated LPMφs were stained with mAbs for mouse F4/80, CD11b, CD11c, CD80, CD86, PD-L1, PD-L2, MHC class II, Gr-1, CD70, CD103, TLR2, TLR4, CD115, or their isotype-matched control Abs (purchased from BD Pharmingen or eBiosciences [San Diego, CA]) for 20 min at 4°C. After staining, cells were washed with PBS, stained with propidium iodide, and analyzed using a FACSCalibur system. CellQuest (BD Biosciences, San Jose, CA) or Flowjo (TreeStar, Ashland, OR) software was used for data analysis.

Migration assay

LPMφ migration ability was assessed using the Transwell system (24-well plate, 6-μm pore; Corning Glass, Corning, NY), as described previously (18). Recombinant mouse MCP-1 and control medium were added to the lower chamber at a final volume of 600 μl. In some experiments, anti-MCP-1 or isotype-matched control Abs (R&D systems) were added to the lower chamber with rMCP-1. LPMφs were added to the upper chamber in a final volume of 100 μl. These chambers were separated by a 6-μm-pore size membrane. Cells were allowed to migrate for 4 h at 37°C. Migrated cells in the lower compartments were collected and counted by FACSCalibur.

Quantitative RT-PCR

Total RNA was isolated from macrophages using an RNeasy Micro Kit (Qiagen, Valencia, CA). CDNA was synthesized with the Quantitect RT Kit (Qiagen). For quantitative RT-PCR, TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays for murine IL-10, CCR2, and CX₃CR1 (Applied Biosystems, Foster City, CA) were used. PCR amplifications were conducted in a thermocycler DNA Engine (OPTICON2; MJ Research, Cambridge, MA). Relative quantifications were achieved by normalization to the values of the β-actin gene.

Murine dextran sulfate sodium-induced colitis model

Colitis was induced in WT and MCP-1^{-/-} mice by intake of 2.0% dextran sulfate sodium (DSS; m.w. 50 kDa; BioResearch, Yokohama, Japan) ad libitum for 5 d, followed by regular drinking water until 7 d. Body weight and clinical findings were monitored every day during the experiment. Mice were sacrificed at day 7, and colonic tissues were removed. Resected colonic tissues were assessed macroscopically. For cytokine analysis, LPMφs were isolated from animals with induced colitis.

Parabiosis experimental design

To assess the requirement for LPMφs, we used a parabiosis system between WT (Ly5.1) and MCP-1^{-/-} (Ly5.2) mice (19). Sex-matched mice were anesthetized before surgery, and incisions were made in the skin on the opposing flanks of the donor and recipient animals. Surgical sutures were used to bring the body walls of the two mice into direct physical contact. The outer skin was then attached with surgical staples. Mice were sacrificed on days 14–21, and splenic and colonic tissues were removed. CD3⁺ T cells and LPMφs were assessed using flow cytometry.

Statistical analysis

The data are expressed as mean ± SEM. Comparisons of the data were performed using a nonparametric Mann-Whitney *U* test. *p* < 0.05 was accepted as statistically significant.

Results

CD11b⁺ LPMφs show an anti-inflammatory phenotype and spontaneously produce a large amount of MCP-1

It has been reported that LPMφs reveal M2-like anti-inflammatory phenotypes in terms of producing cytokines (2). In the current study, we first confirmed the cytokine producing profile of LPMφs. As a control, macrophages and splenic macrophages (SPMφs),

which show M1-like proinflammatory cytokine producing ability, were used (2). Consistent with these recent findings, LPM ϕ s produced higher IL-10, but less TNF- α compared with SPM ϕ s (Fig. 1A). In addition, we found that LPM ϕ s, but not SPM ϕ s, spontaneously produced large amounts of MCP-1 (Fig. 1B). MCP-1 plays a role in recruitment of monocytes or macrophages to inflamed tissue. Therefore, we considered that hyperproduction of MCP-1 from resident CD11b $^+$ LPM ϕ s in a steady state might play a role in the regulatory function of LPM ϕ s.

Murine DSS-induced colitis is exacerbated in MCP-1 $^{-/-}$ compared with WT mice

To determine the functional role of MCP-1 for gut homeostasis, we examined its role in DSS-induced colitis in WT and MCP-1 $^{-/-}$ mice (Fig. 2A). The body weight started to decline from day 4 after the intake of DSS in WT and MCP-1 $^{-/-}$ mice. However, after day 7 the body weight loss was exacerbated significantly in the MCP-1 $^{-/-}$ mice (Fig. 2B; 85.41 \pm 5.3% in WT versus 76.68 \pm 3.0% in MCP-1 $^{-/-}$; $p < 0.01$ at day 7). Macroscopic findings revealed that the shortening (52.75 \pm 3.8 cm in WT versus 49.0 \pm 4.3 cm in MCP-1 $^{-/-}$; $p < 0.05$ at day 7) and bleeding of the colon were markedly more severe in MCP-1 $^{-/-}$ than in WT mice (Fig. 2C, 2D).

BMM ϕ s from WT and MCP-1 $^{-/-}$ mice show similar production levels of IL-10 and TNF- α

DSS-induced colitis was exacerbated in MCP-1 $^{-/-}$ mice; therefore, MCP-1 may play some role in the negative regulation of host immunity. To confirm this, we focused on the anti-inflammatory role of intestinal macrophages that produce cytokines IL-10 and MCP-1. BM CD11b $^+$ cells from WT and MCP-1 $^{-/-}$ mice were differentiated into macrophages with M-CSF, and differentiated macrophages were stimulated with heat-killed *E. faecalis* (MOI = 100) for 24 h (Fig. 3A). BMM ϕ s differentiated from WT and MCP-1 $^{-/-}$ mice showed similar production levels of IL-10 and TNF- α in response to *E. faecalis*. These results suggest that MCP-1 deficiency does not affect differentiation into anti-inflammatory intestinal macrophages.

CD11b $^+$ LPM ϕ s from MCP-1 $^{-/-}$ mice produce less IL-10 than those from WT mice

Because MCP-1 deficiency did not affect macrophage differentiation, we examined CD11b $^+$ LPM ϕ s from WT and MCP-1 $^{-/-}$ mice. CD11b $^+$ LPM ϕ s from both groups of mice were stimulated with *E. faecalis* for 24 h, and production of IL-10 and TNF- α in

culture supernatant was measured. CD11b $^+$ LPM ϕ s from MCP-1 $^{-/-}$ mice produced less IL-10 than did WT mice in response to *E. faecalis* ($p < 0.05$; Fig. 3B). In contrast, the production level of TNF- α in CD11b $^+$ LPM ϕ s from MCP-1 $^{-/-}$ mice was similar to that from WT mice. These results suggest that MCP-1 modulates the composition of resident intestinal macrophages or the production of IL-10 in LPM ϕ s.

CD11b $^+$ CD11c $^-$ LP cells can be separated into two subsets, and CD11b $^+$ F4/80 $^+$ CD11c $^-$ SSC lo LPM ϕ s are reduced significantly in MCP-1 $^{-/-}$ mice

Because the production level of IL-10 in CD11b $^+$ LPM ϕ s was decreased in MCP-1 $^{-/-}$ mice, we hypothesized that the phenotypes of LPM ϕ s themselves might be changed in MCP-1 $^{-/-}$ mice. To confirm this hypothesis, we tried to classify in detail the phenotypes of CD11b $^+$ intestinal macrophages. When flow cytometry of gated CD11b $^+$ CD11c $^-$ LP cells from WT mice was expanded into side scatter (SSC) and forward scatter (FSC), CD11b $^+$ CD11c $^-$ LP cells could be separated into two subsets (Fig. 4A). CD11b $^+$ CD11c $^-$ SSC hi and SSC lo LP cells expressed F4/80, a typical macrophage marker, on their cell surface. CD11b $^+$ F4/80 $^+$ CD11c $^-$ SSC hi LP cells expressed MHC class II, CD80 and coinhibitory molecule PD-L1, while they lacked expression of CD86 and TLRs. This phenotype was similar to the previously identified typical intestinal macrophage subset, which seemed to be anti-inflammatory macrophages. Alternatively, CD11b $^+$ F4/80 $^+$ CD11c $^-$ SSC lo LP cells robustly expressed CD86, Gr-1 (Ly-6G), and TLR2, and to a lesser extent PD-L2, CD70, and CD103, in addition to the markers expressed on CD11b $^+$ F4/80 $^+$ CD11c $^-$ SSC hi LP cells (Fig. 4B). We stained these two subsets in May-Grunwald-Giemsa, and both subsets showed macrophage-like morphology that had a large cytoplasm and non-lobular nuclei (Fig. 4C). We defined CD11b $^+$ F4/80 $^+$ CD11c $^-$ SSC hi LP cells as LPM ϕ 1 and CD11b $^+$ F4/80 $^+$ CD11c $^-$ SSC lo LP cells as LPM ϕ 2. LPM ϕ 2 were reduced significantly in MCP-1 $^{-/-}$ mice (Fig. 4A).

LPM ϕ 2 but not LPM ϕ 1 are migrated by MCP-1

LPM ϕ 2 were reduced significantly in MCP-1 $^{-/-}$ mice; therefore, we confirmed the MCP-1 dependency of this macrophage subset. We examined the CD11b $^+$ F4/80 $^+$ CD11c $^-$ LPM ϕ migration ability by MCP-1 using the Transwell system, and the mRNA transcription of CCR2 and CX $_3$ CR1 in CD11b $^+$ F4/80 $^+$ CD11c $^-$ LPM ϕ s was tested using quantitative RT-PCR. As a result of the migration assay, LPM ϕ 2 but not LPM ϕ 1 migrated in response to

FIGURE 1. CD11b $^+$ LPM ϕ s showed an anti-inflammatory phenotype and spontaneously produced a large amount of MCP-1. **A**, CD11b $^+$ SPM ϕ s and LPM ϕ s (1×10^5 cells) from WT mice were stimulated with heat-killed *E. faecalis* (MOI = 100) for 24 h. The amount of IL-10 and TNF- α in the culture supernatants was measured using a CBA kit. Data are expressed as the mean \pm SEM from three independent experiments. ** $p < 0.01$, compared with SPM ϕ s (Mann-Whitney *U* test). **B**, CD11b $^+$ SPM ϕ s and LPM ϕ s (1×10^5 cells) from WT mice were cultured for 24 h. The amount of MCP-1 in the culture supernatants was measured using a CBA kit. Data are expressed as the mean \pm SEM from three independent experiments. * $p < 0.05$, compared with SPM ϕ s (Mann-Whitney *U* test).

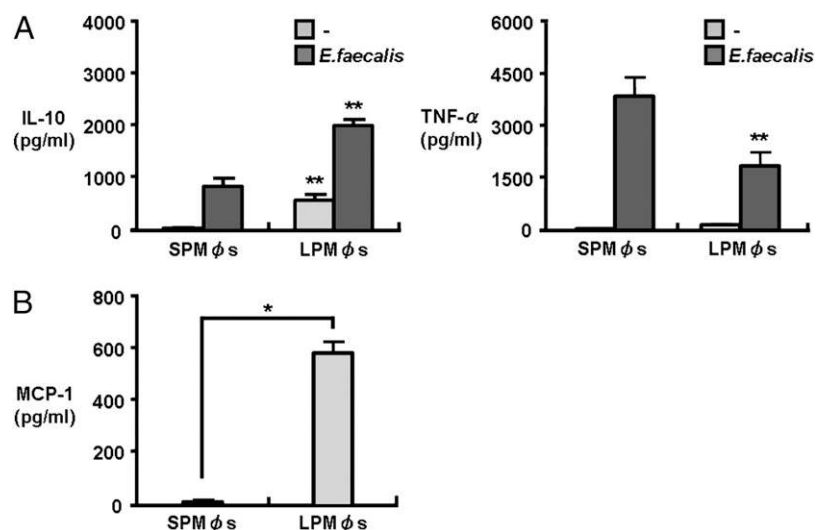
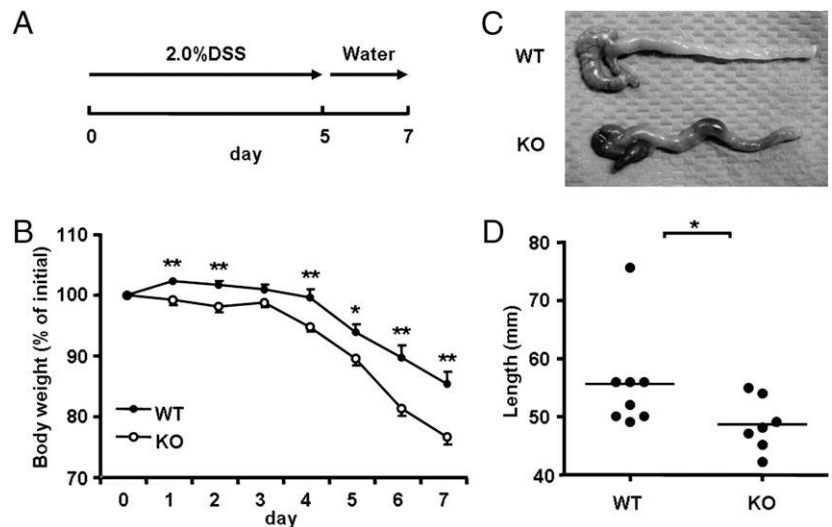


FIGURE 2. Murine DSS-induced colitis was exacerbated in MCP-1^{-/-} mice (knockout [KO]) compared with WT mice. **A**, Murine DSS-induced colitis protocol. **B**, Body weight loss in WT mice with DSS-induced colitis (open circle, $n = 8$) or MCP-1^{-/-} (KO) mice (closed circle, $n = 7$). The data were collected from two independent experiments. * $p < 0.05$; ** $p < 0.01$, compared with WT mice (Mann-Whitney U test). **C**, Macroscopic findings of colons from DSS-induced colitis in WT (upper) or MCP-1^{-/-} (lower) mice. **D**, Colon length in DSS-induced colitis in WT mice or MCP-1^{-/-} mice. The data were collected from two independent experiments. * $p < 0.05$, compared with WT mice (Mann-Whitney U test).



MCP-1 in a concentration-dependent manner (Fig. 5A). Consistent with this result, CCR2, a receptor of MCP-1, was transcribed markedly higher in the LPM ϕ 2 subset than in LPM ϕ 1 (Fig. 5B). Alternatively, CX₃CR1 mRNA transcription was higher in LPM ϕ 1 (Fig. 5B). These results suggest that LPM ϕ 2, but not LPM ϕ 1, is derived from recruited monocytes and localized to the intestinal LP, in an MCP-1-dependent manner.

LPM ϕ 2 is a major source of IL-10 in LPM ϕ s

As shown in Fig. 3B, LPM ϕ s in MCP-1^{-/-} mice produced less IL-10 compared with that in WT mice. To identify which subset of intestinal macrophages was the major source of IL-10, we examined the mRNA transcription and secretion of IL-10 by LPM ϕ 1 and LPM ϕ 2. As a result, the level of bacteria-induced IL-10 mRNA transcripts was markedly higher in LPM ϕ 2 than in LPM ϕ 1 (Fig. 5C). Consistent with mRNA transcription, LPM ϕ 2 produced a large amount of IL-10 in response to *E. faecalis* compared with that in LPM ϕ 1 (Fig. 5C). These results suggest that LPM ϕ 2 is a major source of IL-10.

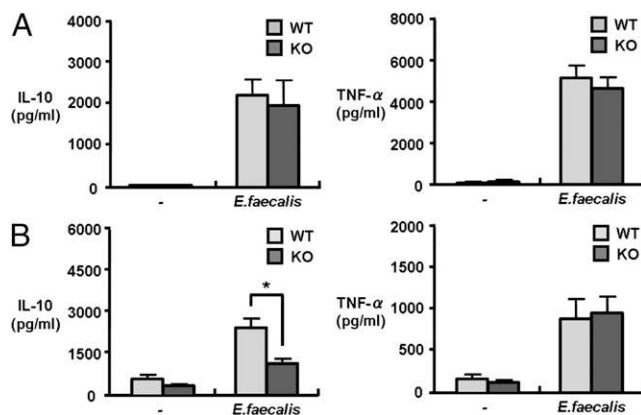


FIGURE 3. CD11b⁺LPM ϕ s from MCP-1^{-/-} mice produced less IL-10 than those from WT mice. **A**, BMM ϕ s (1×10^5 cells) from WT and MCP-1^{-/-} mice (KO) were stimulated with *E. faecalis* for 24 h. The amount of IL-10 and TNF- α in the culture supernatants was measured using a CBA kit. Data are expressed as the mean \pm SEM from three independent experiments. **B**, CD11b⁺LPM ϕ s (1×10^5 cells) from WT and MCP-1^{-/-} mice (KO) were stimulated with *E. faecalis* for 24 h. The amount of IL-10 and TNF- α in the culture supernatants was measured using a CBA kit. Data are expressed as the mean \pm SEM from three independent experiments. * $p < 0.05$, compared with levels in WT mice (Mann-Whitney U test).

Composition of LPM ϕ 2 is impaired in MCP-1^{-/-} mice and LPM ϕ 2 are derived from peripheral blood

To clarify the role of MCP-1 in the composition of resident intestinal macrophages, we used a parabiosis system (Fig. 6A). In the spleen and colon, CD3⁺T cells were mutually mixed, and detectable numbers of donor-derived (CD45.1⁺) cells were observed in MCP-1^{-/-} recipient (Ly5.2; Fig. 6B). A sufficient number of donor-derived (CD45.1⁺) cells were also observed in LPM ϕ 1. However, the number of donor-derived (CD45.1⁺) LPM ϕ 2 was decreased markedly in MCP-1^{-/-} recipient (Ly5.2). These results suggest that MCP-1-dependent monocyte recruitment from the peripheral blood is important for LPM ϕ 2 composition in the steady state.

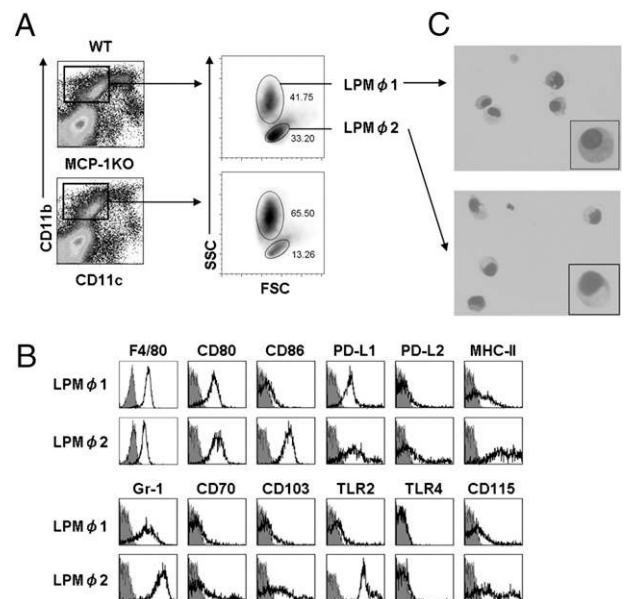


FIGURE 4. CD11b⁺CD11c⁻ LP cells were separated into two subsets, and CD11b⁺F4/80⁺CD11c⁻SSC^{lo} LPM ϕ s were reduced significantly in MCP-1^{-/-} mice. **A**, Flow cytometry of gated CD11b⁺CD11c⁻ LP cells from WT mice expanded into SSC and FSC. CD11b⁺F4/80⁺CD11c⁻SSC^{hi} LP cells were defined as LPM ϕ 1 and CD11b⁺F4/80⁺CD11c⁻SSC^{lo} LP cells as LPM ϕ 2. **B**, LPM ϕ 1 and LPM ϕ 2 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 three independent experiments. **C**, May-Grunwald-Giemsa staining of CD11b⁺F4/80⁺CD11c⁻SSC^{hi} (LPM ϕ 1) and CD11b⁺F4/80⁺CD11c⁻SSC^{lo} (LPM ϕ 2) cells.

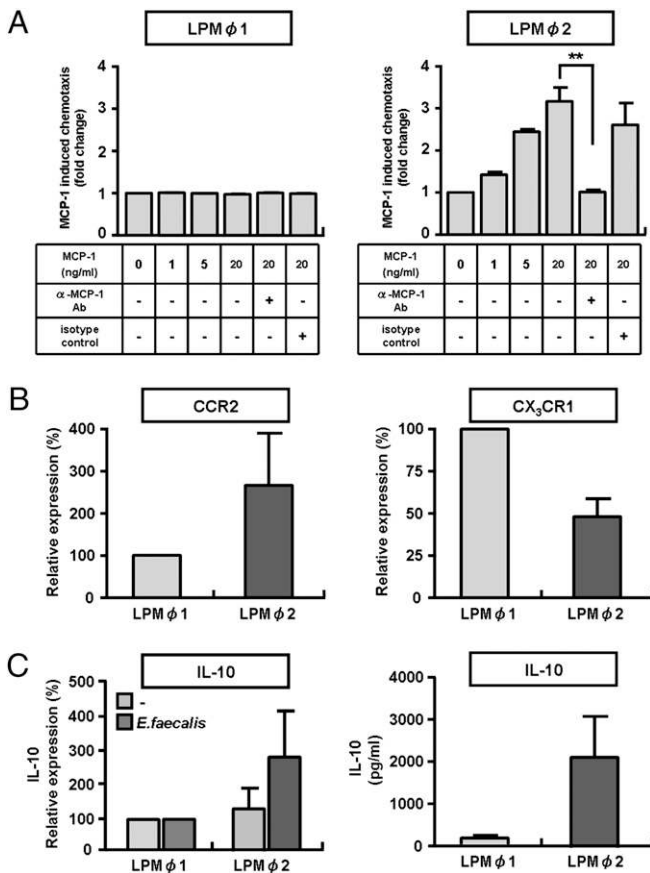


FIGURE 5. LPMφ2 is an intestinal macrophage subset that migrates toward MCP-1, and is a major source of IL-10 in LPMφs. *A*, CD11b⁺ LPMφs migration was assessed using the Transwell system. Numbers of migrated cells in the lower compartments were counted by FACSCalibur. Data are expressed as the mean ± SEM from three independent experiments. ***p* < 0.01 compared with anti-MCP-1 Ab (Mann-Whitney *U* test). *B*, Levels of CCR2 and CX₃CR1 mRNA expression in sorted and LPMφ2 macrophages were analyzed by quantitative RT-PCR and shown as relative percentages of the levels in LPMφ1. *C*, Sorted LPMφ1 and LPMφ2 from WT mice were stimulated with *E. faecalis* for 24 h. Levels of IL-10 mRNA expression were shown as relative percentages of the levels in LPMφ1, and the amounts of IL-10 in the culture supernatants were measured using a CBA kit.

IL-10-producing CD11b⁺ LPMφ recruitment is disturbed in DSS-induced colitis in MCP-1^{-/-} mice

To identify the cause of exacerbation of colitis in MCP-1^{-/-} mice, we measured the level of IL-10 production by CD11b⁺ LPMφs during inflammation. IL-10 production from CD11b⁺ LPMφs in WT mice

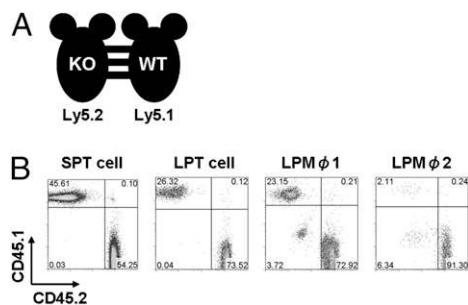


FIGURE 6. Composition of LPMφ2 was impaired in MCP-1^{-/-} mice, and LPMφ2s were derived from peripheral blood. *A*, Parabiosis experimental design. *B*, Flow cytometry of splenic CD3⁺ T cells (SPT cell), colon LP CD3⁺ T cells (LPT cell), and LPMφ1 and LPMφ2 in MCP-1^{-/-} mice stained for CD45.1 and CD45.2.

increased significantly according to the progression of colitis, whereas IL-10 production from CD11b⁺ LPMφs in MCP-1^{-/-} mice increased less than that in WT mice (Fig. 7A). When we examined the ratio of LPMφ1 and LPMφ2 in WT and MCP-1^{-/-} mice in the inflammatory state, the ratio of LPMφ2/ LPMφ1 in WT mice increased significantly, but that in MCP-1^{-/-} mice increased less in the inflammatory state (Fig. 7B). These results suggested that DSS-induced colitis was exacerbated in MCP-1^{-/-} mice because of impaired recruitment of IL-10-producing LPMφ2.

Discussion

MCP-1 plays an important role in the recruitment of monocytes and macrophages from the bloodstream to inflamed tissue. It has been reported that the major source of MCP-1 in inflamed intestinal mucosa is epithelial cells (20), but we demonstrated that intestinal resident macrophages also produced MCP-1, even without inflammation.

In several rodent disease models, MCP-1-dependent infiltrated macrophages are considered to contribute to pathogenesis (10–12). Alternatively, it has been demonstrated that intestinal macrophages contribute uniquely to the maintenance of gut homeostasis (5–7). Whether recruited intestinal macrophages exacerbate intestinal inflammation or contribute to gut homeostasis remains unknown. Intestinal epithelial cells are thought to be a major producer of MCP-1 in the intestine, both in a steady state and during inflammation (20). In addition to these previous reports, we found that LPMφs spontaneously produced significant levels of MCP-1 in a steady state as well as intestinal epithelial cells did. Thus, this spontaneously produced MCP-1 by both cell types may play an important role in the normal composition of LPMφs. We found that LPMφs could be separated into two subsets, LPMφ1 and 2, according to the fluorescence intensity of side-scattered plots in flow cytometric analysis. The LPMφ2 subset, which expressed CCR2 (MCP-1 receptor), was diminished in MCP-1^{-/-} mouse LP, even in the normal state. Thus, endogenous MCP-1 may contribute to the composition of resident intestinal macrophages. As previously reported, intestinal macrophages have unique characteristics, as do anti-inflammatory macrophages; they produce a large amount of IL-10, but not IL-12 and IL-23, in response to enteric bacteria (7). Furthermore, intestinal macrophages can promote Treg development (8). In this regard, intestinal macrophages

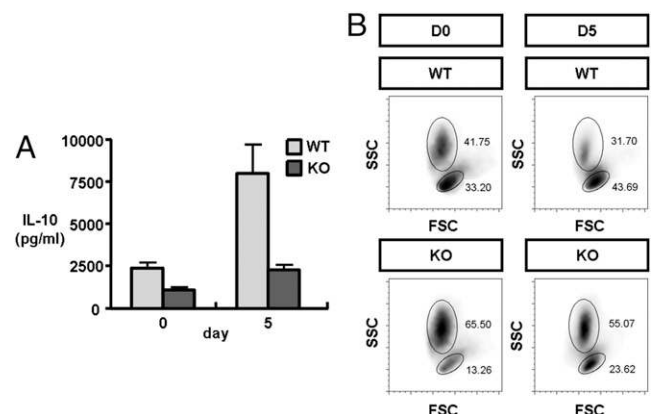


FIGURE 7. IL-10-producing CD11b⁺ LPMφs recruitment was disturbed in DSS-induced colitis in MCP-1^{-/-} mice. *A*, CD11b⁺ LPMφs were isolated from WT and MCP-1^{-/-} mice (KO) in DSS-induced colitis by MACS. CD11b⁺ LPMφs were stimulated with *E. faecalis* (MOI = 100) for 24 h. The amount of IL-10 in the culture supernatants was measured using a CBA kit. *B*, Flow cytometry of gated CD11b⁺ F4/80⁺ CD11c⁻ cells expanded into SSC and FSC in DSS-induced colitis.

in MCP-1^{-/-} mice produced a lesser amount of IL-10 compared with that in WT mice.

In addition to the important role of MCP-1 as a chemoattractant, it has been reported that it can modulate the differentiation of dendritic cells and T cells (21, 22). Dendritic cells generated in the presence of MCP-1 display markedly reduced production of IL-12, but not IL-10, in response to CD40L. MCP-1 promotes differentiation of naive T cells into Th2 cells. However, in the current study, MCP-1 did not modulate the differentiation of macrophages. We examined the direct effect of MCP-1 on IL-10 production from LPMφs; however, MCP-1 did not change IL-10 production from MCP-1^{-/-} LPMφs (data not shown). These findings suggest that MCP-1 does not affect IL-10 production directly, but may contribute to recruitment of IL-10-producing macrophage subsets. Analysis of cytokine production of LPMφ1 and 2 subsets revealed that LPMφ2, which expresses CCR2, produced a larger amount of IL-10 than did LPMφ1.

The classification of monocytic cell lineage has been studied for the past 20 y and has provided insights into functional human and mouse monocyte subsets (1). A previous study has identified that monocytes in mice can be separated into two subsets, CCR2⁺CD62L⁺CX3CR1^{mid} and CCR2⁺CD62L⁺CX3CR1⁺⁺, according to their expression of CCR2, CD62L, Ly6C, and CX3CR1 (23, 24). The CCR2⁺CD62L⁺CX3CR1^{mid} monocyte subset can migrate toward inflammatory lesions in response to locally produced MCP-1. Therefore, this subset has been called *inflammatory monocytes*, which infiltrate inflammatory sites. However, functional roles have not been identified for infiltrated monocytes or newly recruited macrophages for perpetuation or termination of inflammation. Infiltration of mononuclear cells has been observed in several inflamed tissues, whereas infiltrated mononuclear cells play an important role in wound repair (2).

LPMφ2 that produced IL-10 also showed CCR2⁺CX3CR1^{mid} inflammatory monocyte-like phenotypes. An in vitro migration assay showed that LPMφ2, but not LPMφ1, migrated toward MCP-1 in a concentration-dependent manner. An in vivo parabiosis system also implied that LPMφ2 was recruited from peripheral blood in an MCP-1-dependent manner. Therefore, we hypothesized that LPMφ2 is an intestinal macrophage subset that is recruited by MCP-1, and it may contribute to gut homeostasis by producing IL-10 in a steady state and in inflammation. In other words, IL-10-producing LPMφ2 may be recruited by MCP-1 to compose resident intestinal macrophages or to inhibit excess immune responses and terminate inflammation. IL-10-producing LPMφ2s were increased significantly during colitis development. Moreover, IL-10 production by whole CD11b⁺ LPMφs was significantly lower in MCP-1^{-/-} mice than in WT mice because of impairment of LPMφ2 recruitment. Consistent with our hypothesis, immune homeostasis in MCP-1^{-/-} mice is disrupted by impaired migration of MCP-1-dependent suppressive macrophages (e.g., LPMφ2).

In conclusion, we identified that endogenous MCP-1 contributes to intestinal macrophage composition, and MCP-1-dependent intestinal macrophages (e.g., LPMφ2) may play an important role in the maintenance of gut homeostasis in the steady state, and in the termination of excess inflammatory responses in the intestine, by producing the regulatory cytokine IL-10.

Acknowledgments

We thank T. Nakai and K. Arai (Keio University) for technical assistance.

Disclosures

The authors have no financial conflicts of interest.

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