Commensal Gram-positive bacteria initiates colitis by inducing monocyte/macrophage mobilization

Y Nakanishi^{1,2}, T Sato^{1,3} and T Ohteki^{1,2}

Breakdown of the intestinal epithelial layer's barrier function results in the inflow of commensal flora and improper immune responses against the commensal flora, leading to inflammatory bowel disease (IBD) development. Using a mouse dextran sodium sulfate (DSS)-induced colitis model, we show here that commensal Gram-positive bacteria trigger the mobilization of inflammatory monocytes and macrophages into the colon. Monocytes/macrophages are major producers of tumor necrosis factor- α (TNF- α), a representative cytokine that aggravates colitis. Notably, pretreating mice with vancomycin, which eliminated Gram-positive bacteria, particularly the Lachnospiraceae family, significantly reduced the severity of the colitis by selectively blocking the recruitment of monocytes/macrophages, but not of other cells. Importantly, vancomycin treatment specifically downregulated the colonic epithelial cell (cEC) expression of C-C chemokine receptor type-2 (CCR2) ligands, which are critical chemokines for monocyte/macrophage mobilization into the inflamed colon. Collectively, these results provide previously undiscovered evidence that Gram-positive commensal bacteria induce colitis by recruiting colitogenic monocytes and macrophages. Our findings may lead to new avenues of treatment for IBD.

INTRODUCTION

The commensal bacteria in the intestine benefit the host by helping in vitamin synthesis, digestive absorbance, and by acting as a barrier against pathogenic bacteria.¹ In addition, commensal bacteria are essential for fully mature immune responses. In germ-free mice, gut-associated lymphoid tissues such as Peyer's patches, mesenteric lymph nodes, and isolated lymphoid follicles are underdeveloped.² Many studies have associated specific bacteria with particular immunological outcomes, such as T- and B-cell responses. The anaerobic, noncultivable *Segmented filamentous bacteria* stimulate Th17 development in the intestine.³ Human commensal *Bacteroides fragilis*, as well as mouse or human indigenous *Clostridium* species, facilitates regulatory T-cell differentiation and interleukin (IL)-10 production in the gut.^{3,4} While commensal bacteria in the intestine are essential in immune system development, there is growing evidence that dysregulated immune responses against commensal bacteria lead to inflammatory bowel diseases (IBDs), including ulcerative colitis and Crohn's disease. In most spontaneous colitis models, including the IL-10^{-/-} mouse, antibiotics or a

germ-free state can block the development of colitis.^{5,6} However, the role of commensal bacteria in inducing IBD is not well understood.

Recent studies using 16S rRNA sequencing revealed that the intestinal microbiota in humans consists of 10¹³–10¹⁴ microorganisms and up to 1,150 species,^{7,8} which belong to the Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Verrucomicrobiota, or Fusobacteria phyla.^{9–12} It was recently proposed that there are three predominant variants, or “enterotypes”, dominated by *Bacteroides*, *Prevotella*, and *Ruminococcus*.¹³ These enterotypes are not dictated by nationality, sex, age, or body weight. The microbiota in the large intestine is denser and more diverse than that in the small intestine, and mostly consists of Gram-positive Firmicutes or Gram-negative Bacteroidetes, which are conserved in humans and mice.^{9,10} Although the presence of Gram-negative bacteria, *Bacteroides/Prevotella* or Enterobacteriaceae, in the colon is a risk factor for developing IBD,^{14–16} the role of Gram-positive bacteria in colitis is unknown.

Here, by treating mice with dextran sodium sulfate (DSS)-induced colitis with different antibiotics, we demonstrated that

¹Department of Biodefense Research, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan. ²Japan Science and Technology Agency, Core Research for Evolutional Science and Technology (CREST), Saitama, Japan and ³Japan Science and Technology Agency, Precursory Research for Embryonic Science and Technology (PRESTO), Saitama, Japan. Correspondence: T Ohteki (ohteki.bre@mri.tmd.ac.jp)

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Gram-positive commensal bacteria have a critical role in the development of this colitis. We further showed that the presence of Gram-positive bacteria was required for the mobilization of colitogenic monocytes/macrophages into the colon through the local induction of C–C chemokine receptor type-2 (CCR2) ligands.

RESULTS

Monocyte/macrophage mobilization is required for colitis development

The mobilization of inflammatory cells in the colon is an integral component of IBD. We used a mouse DSS-induced colitis model to examine the type of inflammatory cells mobilized in the inflamed colon. Seven days after the onset of the DSS treatment, large numbers of CD11b⁺CD11c^{int/−} cells had accumulated in the inflamed colon (Figure 1a and Supplementary Figure S1a online). Cell-surface markers and the cell morphology revealed four subsets in this fraction: macrophages (R1: major histocompatibility complex type II⁺ (MHC-II⁺)), monocytes (R2: Ly6C^{high}Ly6G[−]), eosinophils (R3: Ly6G[−]Ly6C^{low}), and neutrophils (R4: Ly6G⁺Ly6C^{int}; Figure 1b). Analysis of these cells' ability to secrete tumor necrosis factor- α (TNF- α), a representative cytokine that contributes to the development of colitis,¹⁷ showed that most of the TNF- α -producing cells were monocytes or macrophages (Figure 1c). This was also the case in inducible nitric oxide synthase (iNOS) (Figure 1c). Both monocytes and macrophages in the inflamed colon expressed CCR2 (Supplementary Figure S1b). *Ccr2*-deficient (*Ccr2*^{−/−}) mice exhibited significantly milder colitis as evaluated by the body weight change after DSS onset, and the monocyte/macrophage recruitment was impaired compared with that in wild-type (WT) littermates (Supplementary Figures S2a and b). In contrast, the numbers of neutrophils and eosinophils in the inflamed colon were comparable between *Ccr2*^{−/−} mice and their WT littermates (Supplementary Figure S2c). In line with our findings, ablating Ly6C^{hi} monocytes by anti-MC21 (CCR2) antibodies has been shown to ameliorate DSS-induced colon inflammation.¹⁸ These results strongly suggested that the mobilization and accumulation of monocytes and macrophages into the inflamed colon have a critical role in the development of colitis.

Gram-positive bacteria trigger monocyte/macrophage mobilization into the inflamed colon

To determine whether a particular group of commensal bacteria triggers the monocyte/macrophage mobilization into the inflamed colon, we treated specific pathogen-free mice with vancomycin or colistin, which preferentially target Gram-positive and Gram-negative bacteria, respectively, before the DSS inoculation.^{19,20} As a positive control, specific pathogen-free mice were also pretreated with a combination of ampicillin, metronidazole, neomycin, and vancomycin (A + M + N + V) to kill a wide spectrum of commensal bacteria. Consistent with a previous report,²⁰ quantitative PCR confirmed that administering vancomycin eliminated the Gram-positive bacteria

genus *Clostridia*, whereas colistin treatment reduced Enterobacteriaceae (Supplementary Figure S3). Although excessive vancomycin doses are reported to diminish some Gram-negative *Bacteroides*,²¹ we found that a single daily dose of 1 g/l increased rather than diminished *Bacteroides/Prevotella* and Enterobacteriaceae (Supplementary Figure S3). Notably, 7 days after the DSS challenge, monocyte/macrophage mobilization and accumulation in the colon was significantly lower in mice in which Gram-positive bacteria had been depleted by vancomycin pretreatment, as compared with non-treated or colistin-treated mice (Figure 2). Colistin pretreatment depleted Enterobacteriaceae but did not affect monocyte/macrophage infiltration into the colon. In contrast, depleting the Gram-positive bacteria reduced the neutrophil accumulation slightly but significantly, whereas it did not profoundly affect the recruitment of eosinophils (Figure 2). These data provided the first evidence that in the development of colitis, Gram-positive rather than Gram-negative commensal bacteria trigger the monocyte and macrophage recruitment into the inflamed colon.

Elimination of Gram-positive bacteria decreases susceptibility to DSS-induced colitis

To evaluate whether administering vancomycin, thereby impairing monocyte/macrophage mobilization, ameliorated colitis, we examined body weight loss and inflammatory cytokine production, and performed histological analysis. We noted that, of seven mice treated with broad-spectrum antibiotics (A + M + N + V), two died from systemic sepsis-like death more quickly than mice in other experimental groups. This might have been because of expansion of multidrug-resistant pathobiont *Escherichia coli*.²² After DSS challenge, the vancomycin-treated mice lost significantly less body weight and colon length (Figures 3a and b), and had lower levels of pro-inflammatory cytokines, such as TNF- α and IL-6, in the colon (Figure 3c) compared with the colistin-treated mice or mice that did not receive antibiotics, suggesting that Gram-positive bacteria are required for the development of severe colitis. Consistent with these observations, pathological changes, such as intestinal wall thickening, massive leukocytic infiltration, crypt loss, and histological score in the colon were reduced in the vancomycin-treated mice compared with the colistin-treated mice or mice that did not receive antibiotics (Figure 3d). Collectively, these findings suggested that vancomycin-sensitive Gram-positive bacteria, but not Gram-negative bacteria, caused the development of colitis, at least in part by inducing monocyte and macrophage mobilization into the colon.

Bacteria of the genus *Clostridia* are reported to facilitate regulatory T-cell differentiation in the colon.²⁰ As expected, vancomycin treatment effectively eliminated the genus *Clostridia* (Supplementary Figure S3). However, short-term (7 days) vancomycin treatment did not affect the frequency of Foxp3-positive regulatory T cells in the colon (Supplementary Figure S4a). Consistent with this observation, mice that had received vancomycin recovered more

Figure 1 Role of monocytes and macrophages in the development of colitis. **(a)** Flow cytometry analysis of colon lamina propria cells at 0 (left) or 7 (right) days after beginning dextran sodium sulfate (DSS) treatment: CD11b⁺CD11c^{int-neg} cells within a CD45.2⁺CD3⁻CD19⁻NK1.1⁻Ter119⁻7-AAD⁻ gate (**Supplementary Figure S1**). **(b)** May-Giemsa staining of sorted each cell type. Original magnification: $\times 400$. **(c)** Intracellular tumor necrosis factor- α (TNF- α) and NOS2 staining on each cell type. For TNF- α staining, lamina propria cells were incubated for 3 h in the presence of Brefeldin A. For inducible nitric oxide synthase (iNOS) staining, cells were stimulated with phorbol 12-myristate 13 acetate for 3 h. Results are representative of at least two-three independent experiments.

quickly than those treated with colistin or no antibiotics (H₂O; **Supplementary Figure S4b**).

Altered microbiota composition after vancomycin treatment

To examine how the composition of commensal Gram-positive bacteria is changed by vancomycin treatment, we used 16S rRNA gene sequencing to survey the bacterial populations in fecal samples from untreated or antibiotic-treated mice. Ecological metrics showed that treatment with vancomycin, but not colistin, markedly diminished the richness, which refers to the amount and diversity, of gut microbiota compared with that in controls, as evaluated by rarefaction curve (data not shown). Analysis of the microbiota at various taxonomic levels

demonstrated that vancomycin administration altered the composition of gut microflora (**Figure 4**). Firmicutes and Bacteroidetes were the most abundant phyla, representing more than 79% of the reads (**Figure 4a**). Interestingly, order-level analyses showed that the majority of Firmicutes were Clostridiales in colistin-treated or control mice, but Lactobacillales in vancomycin-treated mice (**Figure 4a**), suggesting that vancomycin treatment eliminated Clostridiales. Family-level analysis of the meta-genome data revealed that Lachnospiraceae, the most abundant order of Clostridiales in the control and colistin-treated mice, was dramatically reduced in the vancomycin-treated mice (**Figure 4b**). As the meta-genome data only represent the relative abundance of each family and vancomycin treatment substantially reduced

Figure 2 Gram-positive bacteria induce monocyte/macrophage recruitment. **(a)** Flow cytometry analysis of mice treated with the indicated antibiotics at 7 days after beginning dextran sodium sulfate (DSS) treatment. SSC and major histocompatibility complex type II (MHC-II) expression within CD11b⁺ CD11c^{int-nega} cells (upper panel) and Ly6G and Ly6c expression within MHC-II⁺ cells (lower panel) are shown. The number indicates the percentage of CD45.2-positive cells. **(b)** Total number of the indicated cell types from antibiotic-treated mice, assayed 0 or 7 days after beginning DSS treatment. The data are pooled from three independent experiments and presented as mean $\pm$ s.e.m. ($n = 4$ –6 mice per group). * $P < 0.05$; ** $P < 0.01$; n.s., not significant.

the amount of total bacteria (**Supplementary Figure S3**, total bacteria), we performed q-PCR and evaluated the absolute quantities by normalizing the results to the amount of total bacteria (**Supplementary Figure S3**). We confirmed that the absolute number of Lachnospiraceae was greatly decreased. In contrast, although the meta-genome data showed that Lactobacillales was replaced by Clostridiales in the vancomycin-treated mice, the q-PCR analysis revealed that the Lactobacillus population was not expanded by vancomycin treatment (**Supplementary Figure S3**). These results implied that the mobilization of monocytes/macrophages into the inflamed colon that leads to colitis development is triggered by the presence of Gram-positive Lachnospiraceae, rather than being suppressed by *Lactobacillus*.

Gram-positive bacteria stimulate the local production of CCR2 ligands

To further investigate the mechanism by which Gram-positive bacteria trigger monocyte/macrophage recruitment into the

inflamed colon, we adoptively transferred Ly6c⁺ Ly6G[−] inflammatory monocytes, which had been sorted from the bone marrow (BM) of congenic B6.SJL mice (CD45.1⁺ CD45.2[−]), into vancomycin-treated WT mice (CD45.1[−] CD45.2⁺) (**Supplementary Figure S5a**). This system allowed us to skip the step of monocyte egress from the BM and focus on their mobilization into the colon. An *in vitro* transwell assay showed that BM monocytes expressed CCR2 and responded to CCR2 ligands, such as CCL2 and CCL7, but did not respond to the unrelated chemokine CCL11 (**Supplementary Figure S5b**). Two days after the transfer, the number of monocytes in the inflamed colon was dramatically reduced in vancomycin-treated compared with antibiotic-untreated hosts (**Figure 5a**), suggesting that the presence of Gram-positive bacteria in the colon modifies the local environment to induce the recruitment of monocytes and macrophages.

CCR2 expression is required for both monocyte egress from the BM and its entry into the inflamed colon.¹⁸ Antibiotics

Figure 3 Effect of reducing monocytes/macrophages on colitis development. **(a)** Changes in body weight after dextran sodium sulfate (DSS) challenge, in mice treated with the indicated antibiotics or not treated with antibiotics. **(b)** Length of the colon in each group ($n = 5$ mice/group). **(c)** Tumor necrosis factor- α (TNF- α) and interleukin (IL)-6 levels, determined using enzyme-linked immunosorbent assay (ELISA), in distal colon tissue homogenate from mice treated with or without antibiotics. Results are representative of two independent experiments; error bars represent s.e.m. ($n = 3$ mice per group). **(d)** Hematoxylin and eosin staining of the distal colon from each experimental mouse model. Original magnification: $\times 40$. Histological scoring was performed as described in Methods. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Vancomycin-treated mice vs. d7 H₂O- or colistin-treated mice.

treatment did not affect the expression level of CCR2 on the monocytes/macrophages in the inflamed colon (**Supplementary Figure 5c**). Therefore, we hypothesized that Gram-positive bacteria induce CCR2 ligands at the local colonic sites during the development of colitis. To test this theory, we isolated an EpCAM⁺ colonic epithelial cells (cEC)-enriched fraction (**Supplementary Figure S5d**), or CD45⁺ hematopoietic cells, 5 days after DSS treatment, and assayed chemokine mRNA levels (**Figure 5b** and **Supplementary Figure S6**). Importantly, vancomycin did not affect the cEC expression of CCL3, CCL5, or CCL11 but diminished the cEC expression of CCR2 ligands such as CCL2, CCL7, and CCL8 (**Figure 5b**). Chemokine expression was not affected by colistin treatment (**Figure 5b**). The hematopoietic-cell

expression of these chemokines was comparable (less than twofolds) among the experimental groups (**Supplementary Figure S6**). These results indicated that Gram-positive bacteria in the colon stimulate cECs to secrete CCR2 ligands, which recruit monocytes and macrophages, a critical component in developing colitis.

DISCUSSION

Our findings showed that vancomycin-sensitive Gram-positive bacteria, but not Gram-negative bacteria, triggered colitis by inducing monocyte/macrophage recruitment into the colon, when the cEC barrier function was disrupted. Supporting this conclusion, although both *Bacteroides* and *Enterobacteriaceae* increased in vancomycin-treated mice

Figure 4 Assessment of microbiota composition after antibiotics treatment. One day before beginning the dextran sodium sulfate (DSS) challenge, sample DNA was extracted from feces from mice treated with the indicated antibiotics. **(a)** The mean relative abundance of bacteria classified at the phylum or order level ($n=3$ per group). Each color indicates a different phylum; pie pieces within Actinobacteria, Bacteroidetes, and Firmicutes represent different orders as indicated. **(b)** The mean relative abundance of families in the Clostridiales order; each color represents a different family.

(Supplementary Figure S3), the monocyte/macrophage recruitment was impaired. In this context, Lachnospiraceae family was particularly reduced in vancomycin-treated mice.

Irrespective of the reason, the impaired barrier function of cECs is one of the common features that initiate colitis development.²³ As a result of epithelial disruption, commensal bacteria flow into the lamina propria of the colon, thereby activating host immune responses. Supporting this scenario, chronic colitis development is impaired in both cEC-death-resistant IL-10^{-/-} mice and germ-free IL-10^{-/-} mice,^{24,25} suggesting that the impaired cEC barrier function and inflow of commensal bacteria are prerequisites for colitis development even in IL-10^{-/-} mice, a DSS-unrelated IBD model. Our

findings indicated that family Lachnospiraceae triggers monocyte/macrophage mobilization into the inflamed colon, and our analysis of species levels showed that this family contains abundant Lachnospiraceae bacterium A4 and *Butyrivibrio fibrisolvens*, by assessing their actual read numbers (data not shown). The former is of particular interest because it is a flagellated bacterium. Sera from Crohn's disease patients and colitic mice react with the flagellin from Lachnospiraceae bacterium A4, and bacterial flagellin is detected by TLR5, which is expressed at the basolateral site of cECs.^{26,27} On the basis of this background and our findings, we speculate that Lachnospiraceae bacterium A4 is a candidate for initiating the monocyte/macrophage mobilization once the barrier function

Figure 5 Gram-positive bacteria induce CCR2 ligands at local colonic epithelium. **(a)** Frequency of transferred cells in the colon: dot plots depict CD45 and CD45.1 expression after CD3⁺ CD19⁺ NK1.1⁺ Ter119⁺ CD11b⁺ CD11c^{int/-} gating. Results are representative of three independent experiments. **(b)** Chemokine mRNA levels on colonic epithelial cells (cECs) 5 days after dextran sodium sulfate (DSS) challenge were assayed by q-PCR. Data are normalized to GAPDH and represent the average of three mice; the fold induction was compared with as that in day 0, H₂O = 1. For CCL8, the value for mice treated with broad-spectrum antibiotics was set as 1. Results are representative of two independent experiments.

of cECs is disrupted. Supporting this possibility, flagellin is known to induce CCL2 secretion by gut epithelial cells.²⁸ However, Lachnospiraceae bacterium A4 is presently unculturable; therefore, the definitive demonstration of this mechanism and how it is involved in colitis development remain subjects for future study.

A recent study demonstrated that the host inflammatory response in the colon, particularly the iNOS-dependent nitrate production, boosts growth of the Gram-negative bacteria *Enterobacteriaceae*.²⁹ In addition, it has been reported that an expansion of *Enterobacteriaceae* is associated with human IBD.^{30,31} We observed that not only TNF- α but also iNOS was strongly expressed in the infiltrating monocytes and macrophages during inflammation (**Figure 1c**). On the basis of this finding, we speculate that monocyte/macrophage-derived nitrates contribute to the host's commensal composition

shift to a Gram-negative-enriched community, eventually worsening the colitis and sepsis-like disease.

In addition to genetic factors, our results imply that the presence of Gram-positive bacteria is an environmental factor for IBD development. Moreover, combined with antibiotics or with anti-TNF- α antibody, a proven therapeutic for IBD patients,³² new strategies that block any step of monocyte/macrophage mobilization may substantially improve outcomes for these patients.

METHODS

Mice. We obtained C57BL/6 (B6) mice from Clea, B6.SJL-ptprca (B6.SJL) mice congenic at the CD45 locus (CD45.1⁺ CD45.2⁻) from Taconic (Germantown, NY), and B6.Ccr2^{-/-} from Jackson Laboratory (Bar Harbor, ME). All mice were bred with Clea B6 mice at our facility to equilibrate the gut flora. Mice were kept 2–3 per cage

under specific pathogen-free conditions using the Micro-VENT system (Allentown, NJ) and were maintained on sterile chow. All animal experiments were approved by the Institutional Animal Care Committee of Tokyo Medical and Dental University.

Antibiotics treatment and DSS induction of colitis. An antibiotic cocktail was prepared by mixing ampicillin (2 g/l; Sigma, St Louis, MO), neomycin (2 g/l; Nacalai, Kyoto, Japan), metronidazole (2 g/l; Nacalai), and vancomycin (1 g/l; Nacalai). To selectively deplete Gram-positive or Gram-negative bacteria, mice were treated with vancomycin or colistin (2 g/l; Sigma), respectively.¹⁹ Mice received daily doses (500 μ l) of vancomycin, colistin, or the antibiotic cocktail, through intragastric gavage for 7–8 days, and were then given 2–2.5% (wt/vol) DSS (MP Biochemicals) in the drinking water for 7 days. The amount of DSS/water intake was comparable in the different strains and treatment groups of mice.

Intestinal cell preparation and flow cytometry. cEC-enriched cell fractions were obtained as described previously³³ with slight modifications. After the colon was excised and the mesentery carefully cleaned, the colon was opened longitudinally, washed with 5% fetal calf serum in HBSS, and cut into small pieces. The colon segments were incubated in 1 mM DTT-supplemented HBSS for 30 min at 37 °C with gentle shaking. Cells in the supernatants were filtered with a 100- μ m cell strainer and then a 40- μ m nylon mesh. The cEC purity was determined with EpCAM staining (> 85%). Colonic lamina propria cells were isolated as described previously.³⁴ The colon was opened and washed with phosphate-buffered saline (PBS), cut into segments, and shaken in PBS containing 5 mM EDTA. The tissue was thoroughly washed in PBS to remove residual epithelial cells, cut into tiny pieces with scissors, and digested by shaking in PBS containing 0.1% type IV collagenase (Sigma-Aldrich, St Louis, MO) for 30 min at 37 °C. The cells were filtered through a 100- μ m cell strainer washed in 1% fetal calf serum–PBS and stained with a combination of anti-CD11c (N418), anti-CD11b (M1/70), anti-I-A/I-E (M5/114.15.2), anti-CD45.2 (104), anti-Ly6C (AL-21), anti-Ly6G (1A8), anti-CD3 (145-2C11), anti-CD19 (6D5), anti-NK1.1 (PK136), anti-Ter119 (TER119), and 7-AAD after blocking Fc receptors. Antibodies were obtained from Biolegend, San Diego, CA or BD Biosciences, San Diego, CA. To analyze chemokine receptors, cell suspensions were stained with an anti-CCR2 antibody (clone 475301; R&D Systems, Minneapolis, MN). For intracellular staining, cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) or the Foxp3 staining kit (eBioscience, San Diego, CA). The cells were then stained with anti-TNF- α (MP6-XT22), anti-NOS2 (CXNFT), or anti-Foxp3 (FJK-16s; eBioscience) antibodies. Flow cytometry was performed on a FACSCanto II (BD Biosciences). For some experiments, cells were further sorted on a FACSaria III (BD Biosciences). The FlowJo software (Tree Star, Ashland, OR) was used for data analysis.

Histological analysis. Tissue harvested from the distal colon (1 cm from the rectum) was cut into segments, fixed with 10% formaldehyde neutral buffer solution (Nacalai) overnight, embedded in paraffin, sectioned at 5 μ m, and stained with hematoxylin and eosin. Histological scoring was performed using a combined score reflecting inflammatory cell infiltration (score, 0–3) and tissue damage (score, 0–3), as described previously.³⁵ For cell infiltration, the presence of occasional inflammatory cells in the lamina propria cell was assigned a value of 0; increased numbers of inflammatory cells in the lamina propria cell as 1; confluence of inflammatory cells, extending into the submucosa, as 2; and transmural extension of the infiltrate as 3. For tissue damage, no mucosal damage was scored as 0; discrete lymphoepithelial lesions as 1; surface mucosal erosion or focal ulceration as 2; and extensive mucosal damage and extension into deeper structures of the bowel wall as 3. The combined histological score ranged from 0 (no changes) to 6 (extensive cell infiltration and tissue damage).

Cytokine measurement in the colonic tissue segments. Distal colon tissue segments (1 cm) were homogenized with scissors and suspended in 500 μ l HBSS containing 0.1% Triton X-100 and protease and phosphatase inhibitors. Supernatants were collected using centrifugation (10,000 r.p.m., 5 min) and assessed for TNF and IL-6 using enzyme-linked immunosorbent assay (ELISA; both from Biolegend).

Adoptive transfer of BM monocytes. BM was flushed from the tibias and femurs of donor B6.SJL (CD45.1⁺CD45.2[−]) mice. After lysing red blood cells with ACK lysing buffer, the cells were stained with PE/Cy5- or Alexa-647-conjugated antibodies against CD3, CD19, NK1.1, Ter119, I-A/I-E, B220, and Ly6G. Lineage-marker-positive cells were depleted with anti-Cy5/Alexa-647 microbeads and an AutoMACSpro separation system (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The monocytes were then negatively sorted with a FACSaria III by depleting cells expressing c-Kit, Sca-1, CD11c, and SSC^{high} (> 95% Ly6G[−]Ly6c⁺). The purified monocytes (1 $\times$ 10⁶) were transferred intravenously into the tail vein of WT hosts (CD45.1[−]CD45.2[±]) 4 days after the start of DSS treatment. WT hosts were either untreated or pretreated with vancomycin before beginning DSS treatment. Transferred cells in the colon were counted 6 days after beginning DSS treatment.

TaqMan quantitative RT-PCR. To determine chemokine expression levels, the total RNA was extracted from enriched cEC (> 85% EpCAM⁺) using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized using random hexamers and SuperScript III reverse transcriptase (Life Technologies, Carlsbad, CA) according to the manufacturers' instructions. Chemokine PCRs were performed with a LightCycler 480 Probe Master (Roche, Mannheim, Germany). Primer pairs and probes were designed at the Universal ProbeLibrary Assay Design Center at the Roche website (<https://q-pcr.probe-finder.com/organism.jsp>).

Chemotaxis assay. Chemotaxis assays were performed as described previously.³⁶ In brief, chemokine dilutions were added to the bottom well of a 96-well chemotaxis plate (NeuroProbe, Gaithersburg, MD). Sorted BM monocytes (4 $\times$ 10⁴) were added on the top of membrane and allowed to migrate at 37 °C for 3 h, after which cells in the bottom wells were counted under a microscope.

16S rRNA gene-quantitative PCR analysis. 16S rRNA gene-quantitative PCR was performed as previously described.²⁰ Bacterial genomic DNA was isolated from fecal pellets using NucleoSpin Soil (Macherey-Nagel, Duren, Germany). Quantitative PCR analysis was carried out using a LightCycler 480. Relative quantity was calculated by the ΔC_t method and normalized to the amount of total bacteria, dilution, and sample weight. The following primer sets were used: total bacteria, 5'-GGTGAATACGTTCCCGG-3' and 5'-TACGGCTACCTTGTTACGACTT-3'; *Enterobacteriaceae*, 5'-TGCCGTAACCTTCGGGAGAAGGCA-3' and 5'-GAATTAACACATACCTCCACTGCTT-3'; *Clostridium leptum* group, 5'-CCTTCCGTGCCGAGTGA-3' and 5'-GAATTAACACATACCTCCACTGCTT-3';²⁰ *Bacteroides/Prevotella*, 5'-CCTWCGATGGATAGGGGTT-3' and 5'-CACGCTACTTGGCTGGTTTCA-3';³⁷ *Lachnospiraceae*, 5'-ACCGCATAAGCGCACAGC-3' and 5'-GGTTACCTTGTTACGACTT-3'.³⁸

Meta 16S rRNA gene sequencing. Bacteria genomic DNA was isolated as described above. The V1–V2 region of the 16S rRNA gene was amplified using KOD DNA Polymerase (Toyobo, Osaka, Japan) with the primers 5'-AGRGTGTTGATYMTGGCTCAG-3' (27Fmod) and 5'-TGCTGCCTCCCGTAGGAGT-3' (338R).³⁹ The Gel clean-up PCR amplicon was conjugated with the adaptor and barcode for the sequence using the Ion Plus Fragment Library Kit and Ion Xpress Barcode Adaptors (Life Technologies) according to the manufacturer's protocols. Sequencing was performed with the Ion Torrent PGM (Life Technologies). FASTQ files were submitted to the MG-RAST bioinformatics pipeline⁴⁰ for analysis. MG-RAST database

searches used an arbitrary 90% cutoff for order identities using the RDP database, and a 98% cutoff for species-level identification. Relative abundance was analyzed using Excel and was denoted with a pie chart.

Statistical analysis. Normally distributed, continuous variables were compared using two-tailed unpaired *t*-tests and one-way analysis of variance followed by Tukey's *post hoc* test using the Prism software (La Jolla, CA).

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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AUTHOR CONTRIBUTIONS

Y.N. designed and conducted the experiments, T.S. assisted with some experiments, and Y.N. and T.O. analyzed data and prepared the manuscript.

DISCLOSURE

The authors declare no conflict of interests.

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