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Enteric Flora Expands Gut Lamina Propria CX3CR1+ Dendritic Cells Supporting Inflammatory Immune Responses under Normal and Inflammatory Conditions

Jan Hendrik Niess and Guido Adler

CD103 or CX3CR1 surface expression defines distinct dendritic cells (DCs) and macrophages in the murine lamina propria of the colon (cLP). We investigated the surface marker and functional phenotype of CD103+ and CX3CR1+ cLP DCs and their role in transfer colitis. cLP CD11c+ cells were isolated from specific pathogen-free or germ-free mice to elucidate the role of the commensal flora in their development. The cLP CD11c+ cells are a heterogeneous cell population that includes 16% CX3CR1+, 34% CD103+, 30% CD103−CX3CR1− DCs, and 17% CD68+F4/80+CX3CR1−CD11c+ macrophages. All DCs expressed high levels of MHC II but low levels of costimulatory (CD40, CD86, and CD80) and coinhibitory (programmed death ligand-1) molecules. Ex vivo confocal microscopy demonstrated that CX3CR1+CD11c+ cells, but not CD103+ DCs, were reduced in the cLP of germ-free (CX3CR1−GFP) mice. The absence of the enteric flora prevents the formation of transepithelial processes by the CX3CR1+ DCs. CX3CR1+ DCs preferentially supported Th1/Th17 CD4+ T cell differentiation. CD103+ DCs preferentially induced the differentiation of Foxp3-expressing regulatory T cells. The stimulation of cLP DCs with fractalkine/CX3CL1 in the absence of CX3CR1, the CD45RBgreg CD4 transfer colitis was suppressed and associated with reduced numbers of DCs in the mesenteric lymph nodes and a reduction in serum IFN-γ and IL-17. The local bacteria-driven accumulation of CX3CR1+ DCs seems to support inflammatory immune responses. The Journal of Immunology, 2010, 184: 2026–2037.

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Address correspondence and reprint requests to Dr. Jan-Hendrik Niess, Department of Internal Medicine I, University of Ulm, Albert-Einstein-Allee 23, D-89081 Ulm, Germany. E-mail address: jan-hendrik.niess@uniklinik-ulm.de

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Abbreviations used in this paper: cLP, lamina propria of the colon; DC, dendritic cell; DN, double-negative; FCM, flow cytometry; GF, germ-free; LP, lamina propria; MLN, mesenteric lymph node; ODN, oligodeoxynucleotide; pDC, plasmacytoid dendritic cell; PD-L1, programmed death ligand-1; siLP, lamina propria of small intestine; SFP, specific pathogen-free; Treg, regulatory T.

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Materials and Methods

Mice

Inbred C57BL/6J (B6) mice, CX3CR1-GFP (B6.129P-Cx3cr1tm1Liy/J) mice, and transgenic OT-II/RAG−/− mice were bred and kept under specific pathogen-free (SPF) conditions in the animal facility of Ulm University. Germ-free (GF) CX3CR1-GFP mice were generated and screened weekly for viral, bacterial, and fungal contamination. CX3CR1-GFP mice were crossed with Rag−/− (Rag−/−/Mmum) animals to obtain Rag−/−/CX3CR1-GFP animals. All experiments were carried out with 6–12-wk-old mice. The experiments were performed according to the guidelines of the local Animal Use and Care Committee and the National Animal Welfare Law.
DC isolation

DCs were isolated from the cLP, spleen, and MLNs of B6 and heterozygous and homozygous CXCR1-GFP animals.

Colonial lamina propria

Segments of the colon were washed with PBS to remove debris and mucous. The epithelium was removed by incubation at 37°C for 30 min under gentle shaking with 1mM DTT and 1mM EDTA in Ca2+-Mg2+-free PBS supplemented with 1% FCS. The remaining tissue was washed in PBS to remove residual epithelial cells, and the supernatants were discarded. Denuded tissues were cut into 2 x 2-mm pieces and digested with 0.5 mg/ml collagenase type VIII (cat. C0-2139; Sigma-Aldrich, St. Louis, MO) and 5 U/ml DNase (cat. no. 1284932; Roche, Basel, Switzerland) for 2 h at 37°C in RPMI 1640/5% FCS. Supernatants were collected, from which LP lymphocytes were pelleted. LP lymphocytes were resuspended in RPMI 1640 medium containing 40% Percoll (density 1.124 g/dl; cat. no. L-6145; Biochrome, Berlin, Germany). This cell suspension was overlaid onto 70% Percoll and centrifuged for 20 min at 750 g. Viable cells at the 40%/70% interface were collected and washed twice.

Spleen and MLN cells

Single-cell suspensions were aseptically prepared from spleen and MLNs, washed, resuspended in Nycodenz (cat. no. 1002380; Axis-Shield, Oslo, Norway), overlaid with RPMI 1640 medium, and centrifuged at 9°C for 90 s x g for 20 min. Cells in the interface were collected and washed twice. Contaminating T cells, B cells, and NK cells were depleted from this cell population by MACS, using PE-conjugated anti-CD3e mAb 145-2C11 (cat. no. 553064; BD Biosciences, Heidelberg, Germany), PE-conjugated anti-CD19 mAb 1D3 (cat. no. 553739; BD Biosciences), PE-conjugated anti-NK mAb DX5 (cat. no. 150-052-501; BD Biosciences), and anti-PE microbeads (cat. no. 130-048-801; Miltenyi Biotec, Bergisch-Gladbach, Germany). Cells were collected for flow cytometry (FCM) analyses or electronic cell sorting. CD103+, CD4+, CXCR1+, and CXCR1+ CD103+ subsets were sorted using the FACSAria system (BD Biosciences).

CD45RBhi CD4 T cell transfer colitis

CD4 T cell populations were isolated from the spleen of Foxp3-GFP (B6, Cg-Foxp3tm2J) mice by MACS (cat. no. 130-090-860; Miltenyi Biotec). Cells were stained for CD4 and CD45RB with APC-conjugated mAb binding CD4 GK1.5 (cat. no. 17-0041; eBioscience, San Diego, CA) and a biotinylated mAb binding CD45RB hi (cat. no. 553097; BD Biosciences), followed by the second-step reagent Streptavidin-Peridinin Chlorophyll-a Protein-Cy5.5 (cat. no. 551419; BD Biosciences). CD45RBhiFoxp3-GFP+ CD4 T cells from the CD4+ population were enriched using the FACSAria system (BD Biosciences) to a purity >95%. Purified CD45RBhiFoxp3-GFP+ CD4 T cells were injected i.p. (3 x 10⁵ cells/mouse) into congenic RAG-1−/− mice (C57BL/6J). In some experiments, cell transfers were carried out with Foxp3-GFP+ CD4 T cells and nonfractionated CD4 T cells containing CD45RBhi and CD45RBlo cells. The weight of transplanted mice and their clinical condition were monitored twice weekly. Tissue samples for histopathological examination were taken from the large intestine, fixed in neutral-buffered formalin, embedded in paraffin, sectioned on a microtome, mounted on slides, and stained with H&E. Histology of the large intestine was categorized as normal (score 0); mild colitis (score 1), with few inflammatory cells in the LP, stroma edema, and a slight reduction of goblet cells; moderate colitis (score 2), with an intense inflammatory infiltration of the LP, hyperplasia of crypts, and a marked reduction of goblet cells; or severe colitis (score 3), with a spillover of leukocytes beyond the mucosa into deeper layers of the colonic wall, complete loss of goblet cells, distortion of the mucosal architecture, erosions or ulcerations, and crypt abscesses, as previously published (20).

Abs

The following reagents and mAbs from BD Biosciences were used: FITC-conjugated mAb binding B220 RA3-6B2 (cat. no. 553088); PE-conjugated mAb binding CD103 M290 (cat. no. 557495), CD80 16-10A1 (cat. no. 553550), CD86 GL1 (cat. no. 553690), CD70 FR70 (cat. no. 555285), CD11b M1/70 (cat. no. 553309), CD8 53-6.7 (cat. no. 553029), CD51 RMV-7 (cat. no. 551380), or CD103 M290 (cat. no. 557493); and APC-conjugated mAb binding CD11c.
transgene-encoded TCR of OT-II mice). Cells were washed three times with medium and transferred into culture. The indicated DC (1 × 10^6/well) and CD4 T cells (1 × 10^5/well) were cultured in 200-μl flat-bottom microwells in RPMI 1640 medium supplemented with 5% FCS, 2 mM L-glutamine, and antibiotics. Foxp3 staining was performed in some experiments (eBioscience, cat. no. 88-8111-40).

**Cytokine detection by ELISA**

Cytokines in supernatants were detected by a conventional double-sandwich ELISA. The following mAbs (from BD Biosciences) were used for detection and capture: mAb R4-6A2 (cat. no. 551216) and biotinylated mAb XMG1.2 (cat. no. 554410) for IFN-γ, mAb JES6-1A12 (cat. no. 554424), biotinylated mAb JES6-5H4 (cat. no. 554426) for IL-2, mAb TC11-18H10 (cat. no. 555068) and biotinylated mAb TC11-81H4.1 (cat. no. 555007) for IL-17A, and mAb TN3-19.12 (cat. no. 557516) and biotinylated mAb C1150-14 (cat. no. 557452) for TNF-α. We used OptEIA ELISA kits for detection of the IL-10 and -6 (cat. nos. 55-52-52 and 55-52-40; BD Biosciences). IL-22 was detected by the Mouse/Rat IL-22 Quantikine ELISA Kit (R&D Systems). The IL-23 ELISA was purchased from eBioscience (cat. no. 88-7234). Extinction was measured at 405/490 nm on a TECAN microplate-ELISA reader using EasyWin software (both from Tecan, Wetzlar, Germany).

**Statistical analyses**

A one-way ANOVA test (for nonparametric data) and a t test for two unequal variances were used. p < 0.05 was considered statistically significant.

**Results**

**CX3CR1 and CD103 are expressed by nonoverlapping DCs**

Different DCs and macrophages in the intestinal LP, the Peyer’s patches, and the MLN can be distinguished by surface phenotype, localization, cytokine production, and/or their ability to drive T cell differentiation in vitro (22). Particular attention has been focused on LP DCs that express the integrin αE (CD103) or the chemokine receptor CX3CR1, which binds CX3CL1/fractalkine. We determined the distribution of plasmacytoid DCs (pDCs), CD11c+ DCs, and CD103+ DCs in spleen, MLN, cLP, and siLP in wild-type and heterozygous Cx3cr1GFP/+ (mice in which the GFP is inserted into one allele of the Cx3cr1 locus) (23). (NK1.1+ DX5+) NK cell-, (CD3+ T) cell-, and (CD19+) B cell-depleted DC populations were isolated from spleen, MLNs, siLP, and cLP and stained for CD11c, B220, CD4, and CD8 (Fig. 1A). Splenic CD11c+ cells made up 40–50% of the pDCs and DN (CD4−CD8−) DCs, >80% of the CD4 DCs, but <20% of the CD8 DCs. In MLNs, >80% CX3CR1+ cells were found in the pDC population, <30% CX3CR1+ cells were found in the DN DC population, and >75% and 49% CX3CR1+ cells were found in the CD4+ and CD8+ DC populations, respectively (Fig. 1B). Hence, CX3CR1 is expressed by pDCs and conventional DC subsets.

We confirmed that CD8α and DN DCs from spleen and MLNs expressed CD103 (Fig. 1C), as previously reported (24, 25). Distinct CX3CR1 and CD103 expression by DCs raises the possibility that they are expressed by nonoverlapping DCs.

* Purified colonic CD11c+ cells from Cx3cr1GFP/+ mice were stained for CD103 to identify cLP CD11c+ cells that express CD103 and/or CX3CR1. Dot blot analysis of gated cLP and siLP CD11c+ cells revealed that 34% of these cells expressed CD103, 34% expressed CX3CR1, 30% expressed neither CX3CR1 nor CD103, and 2% expressed CX3CR1 and CD103 (Fig. 1D). CX3CR1+CD11c+ cells were then stained for F4/80 and CD68. Dot blot analysis demonstrated that 16% of these cells expressed F4/80 and CD68, 34% expressed CD68, and 49% expressed neither CD68 nor F4/80 (Fig. 1E). Thus, the CD11c cell population includes 16% CX3CR1+, 34% CD103+, and 30% CD103+CX3CR1− DCs and 17% CD68+CX3CR1+CD11c− macrophages. We further analyzed the surface phenotype of CX3CR1+CD103+, CX3CR1−CD103+, and CX3CR1−CD103− cLP DCs by gating on CD68+CX3CR1+, CD103+, and CD103+CX3CR1+DCs (Fig. 1F). CX3CR1−CD103+ and CX3CR1−CD103− DCs showed high MHCI class II; intermediate PD-L1; low CD40, CD80, and CD86; and no αV (CD51) surface expression (Supplemental Fig. 1). Collectively, these data demonstrate that CD11c+ cells include 34% CD103+, 16% CX3CR1+, and 30% CD103−CX3CR1−CD11c− DCs and 17% CD68+CX3CR1+CD11c− macrophages.

**CX3CR1+ DCs are reduced in the LP of GF mice**

We generated GF Cx3cr1GFP/+ mice to test whether microbial stimuli provided by the commensal flora are required for the local accumulation of CX3CR1+ DCs and macrophages in the gut LP. We determined the fraction (percentage of all CD11c+ cells) and absolute numbers (per tissue/per mouse) of CD103+ DCs and the CX3CR1+CD11c− population from cLP, siLP, and MLNs of SPF and (age- and sex-matched) GF mice. Because the formation of transepithelial dendrites depends on TLR engagement and the presence of pathogens (26), we used ex vivo confocal imaging of living tissues to determine whether the formation of transepithelial processes in the gut of GF mice depends on the enteric flora (Fig. 2A). Ex vivo three-dimensional reconstructive confocal imaging of large and small intestinal tissues indicated that CX3CR1+CD11c− DCs and macrophages are reduced in the siLP and cLP of GF animals. The siLP of SPF animals is filled with CX3CR1+ cells beneath the epithelium. In GF animals, intestinal villi are more slender and more uniform between gut segments (Fig. 2A). The number of processes was reduced in the absence of the enteric flora. In Cx3cr1GFP/+ mice, we observed 1.6 ± 0.4 transepithelial processes per villus, but only 0.4 ± 0.2 dendrites per villus in GF Cx3cr1GFP/+ animals (Fig. 2B). In the cLP of SPF animals, CX3CR1+ cells are interconnected with dendrites to form a subepithelial network (Fig. 2, Supplemental Fig. 2). The interconnections between CX3CR1+ cells are reduced in the absence of the flora. In Cx3cr1GFP/+ mice, 3.1 ± 0.8 connections per CX3CR1+ cell were found, whereas 1.1 ± 0.5 connections between CX3CR1+ cells were observed in GF Cx3cr1GFP/+ animals (Fig. 2C).

We analyzed the absolute numbers (per tissue/per mouse) of CD11c+ cells in the siLP and cLP of GF and SPF animals. Total numbers of CD11c+ cells were reduced in the siLP and cLP, particularly in the MLNs, from GF mice (Fig. 2D). We isolated 62.1 × 10^5 CD11c+ cells from the siLP of one SPF mouse. In GF animals, the cell isolation yielded 43.7 × 10^5 CD11c+ cells per small intestine. We isolated 20.8 × 10^5 CD11c+ cells and 21.3 × 10^5 CD11c− cells in SPF and GF animals, respectively. In contrast, the numbers of CD11c+ cells and 21.3 × 10^5 CD11c− cells were reduced in the GF animals (9.9 × 10^5 and 20 × 10^5, respectively). CX3CR1+ cells (p = 0.04) and CX3CR1−CD103− DCs (p = 0.03) were significantly decreased in the siLP of GF animals compared with SPF animals (24.1 × 10^5 and 20 × 10^5, respectively). CX3CR1+ cells were not statistically significant (p = 0.8).

The total numbers of CD11c+ cells in the cLP of GF animals were reduced compared with SPF animals (25.6 × 10^5 and 56.7 × 10^5, respectively). CX3CR1+CD11c+ DCs and macrophages were reduced in GF animals (7.6 × 10^5) compared with SPF animals (19.3 × 10^5). Additionally, CX3CR1−CD103+ DCs were reduced in GF animals (9.9 × 10^5) compared with SPF animals (15.2 × 10^5). Reduced numbers of CD103+ DCs were observed in the cLP of GF animals (18.2 × 10^5) compared with SPF animals (22.3 × 10^5) (Fig. 2F). The difference in total CD103+ DCs was not significantly changed in the cLP (p = 0.5); however, the total numbers of CX3CR1+CD11c− cells were significantly decreased (p = 0.03).

Microbial stimuli induce the migration of DCs from the LP to the MLN. When we analyzed the absolute numbers (per tissue/per mouse) of CD11c+ cells in the MLNs of GF and SPF animals (Fig. 2G). The number of DCs was 7-fold greater in the MLNs from
SPF Cx3cr1^{GFP/+} mice compared with their GF counterparts, whereas the CD103^{+} and CX3CR1^{+} CD11c^{+} populations were 7-fold greater in SPF mice (Fig. 2E). CCR7 controls DC migration from peripheral tissues to the draining lymph nodes. In the cLP, 14% of CD103^{+} cells expressed CCR7. CX3CR1^{+} DCs and macrophages and CX3CR1^{+} CD103^{+} DCs did not express CCR7 (Supplemental Fig. 5). The absence of the enteric flora prevented the expression of CCR7 by CD103^{+} DCs. Because CD103^{+} DCs are reduced in the MLNs but not in the cLP of GF animals, the enteric flora may provide stimuli, inducing the migration of DCs to the draining lymph nodes.

cLP DCs express TLRs

The CX3CR1^{+}, CD103^{+}, and CX3CR1^{−} CD103^{−} DCs were isolated from the cLP, enriched by MACS, and analyzed for TLR expression by FCM. Intracellular staining was carried out for the detection of TLR-9. TLR-2 and -4 expression by cLP DCs was checked by surface staining by gating on F4/80^{+} CD68^{+} CX3CR1^{+},

**FIGURE 1.** CX3CR1 and CD103 are expressed by nonoverlapping cLP DCs. A, CD11c^{+} cells were isolated from the spleen, MLNs, siLP, and cLP. Cells were stained for CD11c, B220, CD4, and CD8α and analyzed by four-color cytofluorometry. Expression of CD4 and CD8α by conventional CD11c^{+} B220^{−} DCs was analyzed. Numbers indicate the percentage of DCs that express CD4 or CD8α. Data from an individual representative mouse (of five individual mice analyzed) are shown. CX3CR1 (B) and CD103 (C) expression by plasmacytoid DCs and CD4^{+}, CD8α^{+}, and CD4^{−} CD8α^{−} DCs from spleen and MLNs of Cx3cr1^{GFP/+} B6 mice was analyzed by multicolor FCM. Filled curves represent the respective negative controls (syngeneic B6 mice). Data from an individual representative mouse (of five individual mice analyzed) are shown. D, cLP CD11c^{+} cells from heterozygous and homozygous Cx3cr1-GFP animals stained for CD103. Numbers indicate the percentage of CD103^{+} or CX3CR1^{+} CD11c^{−} DCs and/or CX3CR1^{+} cells in the gated CD11c^{+} population. Data from an individual representative mouse per group (of five individual mice analyzed) are shown. E, Intracellular CD68 and surface F4/80 expression by CD11c^{+} CX3CR1^{+} cLP cells. F, Surface expression of CD103, CD11b, MHC class II (Ab), CD40, CD80, CD86, PD-L1, CD81, and CCR9 gated on CD103^{+} or CX3CR1^{+} CD11c^{−} DCs and on CD68^{+} F4/80^{+} CX3CR1^{+} DCs from Cx3cr1^{GFP/+} mice analyzed by multicolor FCM. Filled curves represent respective isotype controls. Data from an individual representative mouse per group (of five individual mice analyzed) are shown.
CD103, and CX3CR1+/CD103− DCs. We found that TLR-9 was expressed by all DCs in the cLP (Fig. 3). Reduced TLR-4 and -2 expression was detected compared with CD103+ DCs. Increased TLR-9 and -2 expression by CX3CR1+ DCs isolated from the cLP of SPF and GF animals. Hence, CX3CR1+/CD103− DC subsets differ in their induced cytokine expression profile in response to stimulation with CpG-containing ODN or after CD40 ligation.

CX3CR1+ DCs induce the differentiation of Th1 and Th17 responses in vitro

Because the concentration of proinflammatory cytokines was increased in the supernatant of ODN-stimulated CX3CR1+ DCs compared with CD103+ DCs, we tested whether CX3CR1+ and CX3CR1+/CD103− DCs induce different T cell responses. Antigenic peptide-pulsed DCs were cocultured for 1–5 d with naive (RAG−/−) OT-II CD4 T cells (Fig. 5). CD4 T cells stimulated with CX3CR1+ DCs produced more IL-2 and -6 and IFN-γ. The secreted IL-2 and -6 were consumed by the cultures within 2 d. IFN-γ and IL-10 concentrations increased within a 5-d culture. Furthermore, peptide-pulsed CX3CR1+ DCs triggered significantly more IL-17 production by CD4 T cells than CD103+ and CX3CR1+/CD103− DCs. In contrast, OT-II CD4 T cells stimulated with CD103+ DCs secreted the immunoregulatory cytokine IL-10. We tested whether CD103+ or CX3CR1+ DCs generate Foxp3-expressing Treg cells (Fig. 6). CD4 T cells cocultured with peptide-presenting CD103+, CX3CR1+, or CD103+CX3CR1− DC subsets were harvested and stained for intracellular Foxp3 expression. Many splenic Foxp3+ CD4 T cells
from OT-II RAG−/− mice) primed by cocultured, peptide-pulsed CD103+ DCs developed readily detectable Foxp3 expression. Thus, we confirmed that CD103+ DCs isolated from the cLP support generation of Treg cells (11). The selective expression of CD103 and CX3CR1 by CD68−CD11c+ DCs divides intestinal DCs and macrophages into subsets that induce the differentiation of proinflammatory

FIGURE 3. CX3CR1+ DCs express TLR-9, -4, and -2. A, DCs were isolated from the cLP of Cx3cr1GFP/+ mice. Cells were stained for CD11c, F4/80, CD103, and TLR-4 and -2. An intracellular staining was carried out for the detection of TLR-9. TLR-4 and -2 protein expression was analyzed by surface staining by gating on F4/80+CX3CR1+, CD103−, and CX3CR1+ CD103+ DCs. TLR expression by the indicated DC subset was analyzed by four-color cytometry. Filled curves represent the corresponding isotype controls. Mean fluorescence intensity is presented as mean ± SEM of five individual analyzed Cx3cr1GFP/+ mice for TLR-9 (B), TLR-4 (C), and TLR-2 (D). In the non-parametric Student t test, p < 0.05 was considered statistically significant.

FIGURE 4. CX3CR1+ DCs release proinflammatory cytokines. Sorted cLP CD103+, CX3CR1+, or CD103−CX3CR1− DCs (purity >95%) were stimulated with 10 ng/ml ODN or CD40L-expressing J558 transfectants (1:3 ratio) for 2 to 5 d. Supernatants were collected, and IL-10 (A), IL-22 (B), IL-6 (C), IL-12p40 (D), and IL-23 (E) were determined by ELISA. The mean ± SEM of three independent experiments is shown. In the nonparametric Student t test, p < 0.05 was considered statistically significant.
T cell subsets or Foxp3-expressing T cells. The expression of different surface molecules may reflect the adaptation of mononuclear cells to the local environment associated with distinct subspecializations.

CX3CL1 increases the release of (pro)inflammatory cytokines by LPS-stimulated cLP DCs

To examine the role of CX3CL1 on the production of (pro)inflammatory cytokines by cLP CD11c+ DCs, we stimulated isolated cLP subsets with soluble CX3CL1. We confirmed that CX3CL1-stimulated DCs and macrophages released IL-6 and TNF-α (30). When cLP DCs were stimulated with LPS and CX3CL1, the addition of CX3CL1 increased the release of IL-6 and TNF-α by DCs isolated from B6 animals but not from Cx3cr1GFP/GFP animals (Fig. 7, data not shown). CX3CL1 did not significantly change the release of IL-6 and TNF-α by LPS-stimulated CD103+ and CX3CR1+CD103− DCs. When CX3CL1 was added to ODN-stimulated cLP DCs, it did not significantly change the cytokine secretion pattern (data not shown). The stimulation of cLP DCs with CX3CL1 seems
fractalkine/CX3CL1 increases the release of IL-6 and TNF-α by LPS-stimulated CX3CR1+ DCs. cLP DCs (1 × 10^5/well) were stimulated with 10 ng/ml LPS and/or 100 ng/ml CX3CL1 for 48 h. Supernatants were harvested, and the concentration of TNF-α in supernatants of cultures with CX3CR1+ DCs (A), CX3CR1+ CD103+ DCs (B), or CD103+ DCs (C) were determined by ELISA. Harvested supernatants from cultures with CX3CR1+ DCs (D), CX3CR1+ CD103+ DCs (E), or CD103+ DCs (F) were checked for IL-6 by ELISA. In the Student t test, p < 0.05 was considered statistically significant.

FIGURE 7. Fractalkine/CX3CL1 increases the release of IL-6 and TNF-α by LPS-stimulated CX3CR1+ DCs. cLP DCs (1 × 10^5/well) were stimulated with 10 ng/ml LPS and/or 100 ng/ml CX3CL1 for 48 h. Supernatants were harvested, and the concentration of TNF-α in supernatants of cultures with CX3CR1+ DCs (A), CX3CR1+ CD103+ DCs (B), or CD103+ DCs (C) were determined by ELISA. Harvested supernatants from cultures with CX3CR1+ DCs (D), CX3CR1+ CD103+ DCs (E), or CD103+ DCs (F) were checked for IL-6 by ELISA. In the Student t test, p < 0.05 was considered statistically significant.

to support the release of IL-6 and TNF-α by LPS-stimulated cells. To test the role of CX3CR1–CX3CL1 interactions during colitis, CX3CR1-GFP mice were crossed with RAG-1−/− mice.

**CX3CR1 deficiency is associated with reduced DC numbers in the MLN during transfer colitis**

CD4 CD45RB<sup>high</sup> Foxp3-EGFP<sup>−</sup> T cells were adoptively transferred into (homozygous or heterozygous for Cx3cr1) RAG-1−/−/CX3CR1-GFP animals. In a first set of experiments, we compared the induction of colitis by transfer of equal numbers of sorted CD4<sup>+</sup> CD45RB<sup>high</sup> CD4 T cells, nonfractionated CD4 T cells (containing CD4<sup>+</sup>CD45RB<sup>high</sup> and CD4<sup>+</sup>CD45RB<sup>low</sup> cells), or sorted Foxp3-EGFP CD4 T cells. RAG−/− hosts that received CD4<sup>+</sup>CD45RB<sup>high</sup> Foxp3-EGFP<sup>−</sup> CD4 T cells developed a rapid colitis within 30 d. After transfer of nonfractionated CD4 T cells into RAG−/− hosts, the development of colitis was attenuated compared with hosts that received CD4<sup>+</sup>CD45RB<sup>high</sup> Foxp3-EGFP<sup>−</sup> CD4 T cells. RAG−/− hosts that received Foxp3-EGFP CD4 T cells were protected from the development of colitis as shown by body weight curves and histology (Supplemental Fig. 4). We decided to use the Foxp3-EGFP animals as donor animals for our CD4<sup>+</sup>CD45RB<sup>high</sup> transfer colitis studies. We determined the distribution of DCs in the cLP and MLNs of (homozygous or heterozygous for Cx3cr1) RAG-1−/−/CX3CR1-GFP hosts with transfer colitis. We analyzed the fraction (percentage of all CD11c<sup>+</sup> cells) and absolute numbers (per tissue/ per mouse) of CD11c<sup>+</sup>, CD103<sup>+</sup>, and CX3CR1<sup>+</sup> CD103<sup>+</sup> DCs in heterozygous and homozygous RAG−/−/CX3CR1-GFP hosts with transfer colitis. Significant differences in absolute CD11c<sup>+</sup>, CD103<sup>+</sup>, and CX3CR1<sup>+</sup> CD103<sup>+</sup> cell numbers between RAG−/−/Cx3cr1<sup>GFP/GFP</sup> and RAG−/−/Cx3cr1<sup>GFP/GFP</sup> hosts were not observed in the inflamed cLP (data not shown). When we analyzed the fraction (percentage of all DCs) and absolute numbers (per tissue/ per mouse) in the MLNs of RAG−/− hosts with transfer colitis, CD11c<sup>+</sup> cell numbers were >3-fold reduced in MLNs from CD11c<sup>+</sup>, CD103<sup>+</sup>, and CX3CR1<sup>+</sup> CD103<sup>+</sup> DCs. The local accumulation CX3CR1<sup>+</sup> DCs and macrophages seems to support inflammatory immune responses in the cLP.

**Discussion**

We found that DCs in the cLP are a heterogeneous population comprising CD103<sup>+</sup>, CD11c<sup>+</sup>, and CD103<sup>+</sup> CD11c<sup>+</sup> DCs. The local accumulation CX3CR1<sup>+</sup> DCs and macrophages...
depends on the enteric flora, because reduced numbers of CX3CR1+ cells were found in the LP of GF animals. The CD45RBhigh CD4 T cell transfer colitis was suppressed in CX3CR1-deficient hosts associated with reduced numbers of DCs in the MLN and a reduced increase in serum IFN-γ and IL-17. CX3CR1 is expressed by DCs, macrophages, T cells, intestinal epithelial cells, and NK cells. The CX3CR1+ cell population isolated from the cLP of heterozygous CX3CR1-GFP animals consisted of 44% CD11c-F4/80+, 14% CD11c-F4/80−, 15% CD11c−F4/80+, and 27% CD11c−F4/80− cells (data not shown). The CD11c+CX3CR1+ populations included F4/80+CD68+ DCs and F4/80−CD68− macrophages. Macrophage-DC precursors give rise to monocytes and DCs (31, 32). The development of CX3CR1+ CD11c+ cells depends on the presence of macrophage-CSF receptor ligands (33, 34). Reduced numbers of Gr1low monocytes have been observed in CX3CR1-deficient animals. An increased death or an impaired recruitment of monocytes from the bone marrow to the spleen has been suggested as cause for the reduced monocyte numbers in CX3CR1-deficient animals (35, 36). Soluble fractalkine/CX3CL1 increased the release of IL-6 and TNF-α by LPS-stimulated cLP DCs and macrophages, confirming previous studies (30). CX3CL1 regulates the release of cytokines by DCs and macrophages in a dose-dependent manner (37). The release of CX3CL1 is induced by proinflammatory mediators, such as LPS, TNF-α, IL-1, and IFN-γ (38–40). Binding of CX3CL1 to CX3CR1 activates the NF-κB pathway and induces the release of proinflammatory mediators, such as IL-8 (41). CX3CR1 deficiency is associated with the reduced release of IL-6 and TNF-α and reduced inducible NO synthase production by macrophages and DCs (30). As a consequence, bacterial killing is impaired in CX3CR1-deficient animals with increased Listeria and Salmonella burden after infection compared with B6 animals (13, 36).

Ileal CX3CR1+ DCs survey the intestinal lumen by extending transepithelial processes into the intestinal lumen (13). Reduced numbers of processes were reported in CX3CR1-deficient animals (13, 18). The challenge of mice with pathogens (i.e., Salmonella or Trichuris muris) or oral challenges with LPS facilitate the extension of processes into the intestinal lumen (26, 42). Because reduced numbers of transepithelial processes were observed in the absence of the enteric flora, the enteric flora seems to facilitate dendrite formation. CX3CR1+ DCs are reduced in the cLP and siLP of GF hosts. Axenic animals are characterized by more slender and shorter ileal villi compared with SPF animals (43) in which CX3CR1+ DCs are located. Intestinal villi of GF mice are more uniform compared with SPF animals (44). In rats, the numbers of OX62+ DCs is reduced. Additionally, DCs that express the inducible isoforms of NO synthase are reduced in GF mice and Myd88-deficient animals (45). The expression of activation markers on DCs from GF mice is decreased (46). CX3CR1+ cells in the cLP are interconnected by dendrites to form a network below the epithelium. The formation of dendrites between CX3CR1+ cells is reduced in the absence of the enteric flora.

![FIGURE 8. CX3CR1 deficiency suppresses transfer colitis. A, Mean ± SEM loss of body weight (% of nine individual mice per group. B, Large intestinal tissues from transplanted CX3CR1GFP+/ and CX3CR1GFP/GFP RAG−/− mice were imaged ex vivo by confocal microscopy. C, Histopathological scores of mouse lines transplanted with CD45RBhigh Foxp3-EGFP+ CD4 T cells. In the one-way ANOVA (nonparametric) test, p < 0.05 was considered statistically significant.](http://www.jimmunol.org/Downloadedfrom/2034DENDRITICCELLSINTHECOLONICLAMINAPROPRIA)
We confirmed that CD103+ DCs induce the differentiation of Foxp3+ CD4 Treg cells (10–12). Immunodeficient hosts lacking CD103 develop a serious wasting disease (25) compared with RAG\(_2\)/\_2 hosts. Dynamic interactions between immunosuppressive and -activating DCs and/or macrophages may have evolved in adaptation to the enteric flora to maintain intestinal homeostasis (19). TLR-2 and -9 expression by CX3CR1+ DCs was greater compared with CD103+ DCs; among the studied TLRs, the expression

![FIGURE 9.](Image)

The presence of CX3CR1 increases IL-17 and IFN-\(\gamma\) expression in mice with transfer colitis. A, cLP CD4 T cells were isolated from RAG\(_2\)/\_2 and heterozygous and homozygous CX3CR1-GFP/RAG\(_2\)/\_2 hosts transplanted with CD45RB\_high Foxp3-EGFP B6 mice. The isolated CD4 T cells were stimulated ex vivo with phorbol ester and ionomycin (in the presence of Brefeldin A), surface stained for CD4, fixed, stained for intracellular IL-17A, IFN-\(\gamma\), TNF-\(\alpha\) or IL-10, and analyzed by FCM. The presence of IL-17 (B), IFN-\(\gamma\) (C), and TNF-\(\alpha\) (D) in the serum of CD45RB\_high CD4 T cell-transplanted heterozygous and homozygous CX3CR1-GFP/RAG\(_2\)/\_2 and RAG\(_2\)/\_2 hosts. The serum concentrations were determined by conventional double-sandwich ELISA. Data are presented as median ± 95% quartile in box-and-whisker plots. Each symbol represents an individual mouse. In the one-way ANOVA test, \(p < 0.05\) was considered statistically significant.

### Table II. CD4 T cells recovered from the cLP of RAG\(_{2}\)/\_2/Cx3cr1\_GFP\_GFP and RAG\(_{2}\)/\_2/Cx3cr1\_GFP\_/\_-GFP mice with colitis transplanted with congenic CD45RB\_high CD4 T4 cells

<table>
<thead>
<tr>
<th>Transfers</th>
<th>Total CD4 T cells</th>
<th>IL-17</th>
<th>IFN-(\gamma)</th>
<th>TNF-(\alpha)</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45RB_high CD4 (\rightarrow) RAG(_{2})/_2/Cx3cr1_GFP_GFP</td>
<td>67 ± 14</td>
<td>4.2 ± 1.5 (5.5 ± 1.7%)</td>
<td>12.9 ± 5.7 (20.2 ± 4.5%)</td>
<td>8.3 ± 3.9 (13.6 ± 2.4%)</td>
<td>0.9 ± 0.3 (1.0 ± 0.5%)</td>
</tr>
<tr>
<td>CD45RB_high CD4 (\rightarrow) RAG(_{2})/_2/Cx3cr1_GFP_/_-GFP</td>
<td>166 ± 13</td>
<td>6.5 ± 1.1 (3.4 ± 2.4%)</td>
<td>39.3 ± 8.2 (24.8 ± 1.1%)</td>
<td>20.7 ± 2.9 (14.3 ± 0.6%)</td>
<td>0.6 ± 0.2 (0.5 ± 0.2%)</td>
</tr>
<tr>
<td>CD45RB_high CD4 (\rightarrow) RAG(_{2})/_2/Cx3cr1_GFP_/_-GFP</td>
<td>154 ± 23</td>
<td>6.3 ± 1.7 (4.1 ± 1.3%)</td>
<td>42.0 ± 12.5 (27.6 ± 4.6%)</td>
<td>19.3 ± 10.2 (14.6 ± 1.5%)</td>
<td>0.7 ± 0.3 (0.4 ± 0.1%)</td>
</tr>
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</table>

Mean ± SEM of five individual mice per group are shown. Bold type indicates significant differences in the number and the percentage of cytokine-producing cLP CD4 T cells between homozygous and heterozygous CX3CR1-GFP animals as determined by the two-tailed Student t test. A \(p\) value < 0.05 was considered statistically significant. Differences between all other groups were not statistically significant. The experiment was repeated once with comparable results.

\(\ast\)CD4 T cells recovered from the cLP of indicated RAG\(_{2}\)/\_2 hosts transplanted with \(3 \times 10^7\) B6 CD45RB\_high Foxp3-EGFP CD4 T cells.

\(\ast\)Total numbers of cLP CD4 T cells producing the indicated cytokine after a brief ex vivo stimulation. The percentage of cells within the isolated cLP CD4 T cell population producing the indicated cytokine is shown in parentheses.
of TLR-9 was the highest, which is in accordance with recent studies. These reports described lower TLR expression by CD103+ DCs compared with the CD103− fraction (47). Although surface TLR-4 is expressed by cLP DCs, we observed greater cytokine release by LPS-stimulated cLP DCs and macrophages. CX3CR1+ DCs produce IL-6, -12p40, and −23 in response to CpG-containing ODN. When GF mice are fed bacterial carbohydrate polysaccharide A, CD4+ T cells are activated, resulting in a Th1 response induced by DCs (48). The local accumulation of Th17- and IFN-γ-producing T cells depends on the enteric flora (20), which is driven by commensal-derived ATP and/or the presence of specific microbiota (i.e., Bacteroides) in the intestine (49, 50). The CX3CR1+ DCs were identified as cells that preferentially support the differentiation of Th1/Th17 cells.

The role of IL-17 in the development of colitis is under debate (51–53). Th17 cells are prominent in murine responses to pathogenic Citrobacter (54), Klebsiella (55), or Streptococcus pneumoniae (56). Transgenic mice lacking IL-17A and -17F develop mucocutaneous abscesses in tissues around the oral cavity and nose (57). Th1 cells mediate the host defense to extracellular bacteria. Th17 cell development can be antagonized by products of T cells of the Th1 and/or Th2 lineages (58). The development of IL-17-producing T cells is suppressed and the development of IFN-γ-producing T cells is favored in the absence of TGF-β but in the presence of IL-23 and −17 (59). The commensal-dependent accumulation of CXCR1+ DCs and macrophages supports inflammatory immune responses in the gut. Local effector responses may have developed to prevent the potential harmful impact of the enteric flora on the host. The existence of DCs that drive effector responses and of subsets that control the effectors may have developed in adaptation to challenges provided by the local gut environment.

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Disclosures
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References