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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 increased the release of IL-6 and TNF-α. In the absence of CX3CR1, the CD45RBhigh 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.

Dendritic cells (DCs) program local immune responses by priming naive T cells and driving their differentiation (1, 2). Murine DCs have been divided into plasmacytoid (B220+) and conventional (CD4−CD8−, CD8−CD4+, and double-negative [DN] CD8−CD4−) subsets (3). Although several DC subsets were described as having a specific function, DCs are characterized by high plasticity among subsets. Local tissue-derived factors seem to have an impact on the phenotype and function of DCs (4). In the intestine, DCs reside in the lamina propria (LP) of the small intestine (siLP) and the colon (cLP), isolated lymph follicles, Peyer’s patches, and mesenteric lymph nodes (MLNs) (5). CD103+ (the αε chain of the αEβ7 integrin) siLP DCs are potent inducers of homing receptors (CCR9 and the α4β7 integrin) on (CD4 and CD8) T and IgA+ B cells (6–9). These subsets have the enhanced capacity to induce the differentiation of Foxp3-expressing regulatory T (Treg) cells. In part, this process is driven by the vitamin A metabolite retinoic acid (10–12).

CX3CR1+ DCs are closely associated with the epithelial lining (13). CX3CR1+ binds fractalkine/CX3CL1, which is expressed in a soluble and a membrane-bound form) by intestinal epithelial and endothelial cells (14, 15). In the particular case of the intestine, DCs play a central role in sampling and processing luminal Ags (16). CX3CR1+ DCs extend transepithelial dritides into the gut lumen to sample and process luminal Ags (13). CX3CR1+ DCs initiate the host defense to intestinal pathogens, such as Salmonella (17, 18), as shown by the enhanced susceptibility of CX3CR1-deficient animals to Salmonella infection (13). Interactions of DCs with T cells can mediate adaptive immunity required for the clearance of pathogens. Macrophage-derived IL-10 controls the production of proinflammatory cytokines by CX3CR1+ DCs (19).

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Department of Internal Medicine I, Ulm University, Ulm, Germany

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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-1, programmed death ligand-1; siLP, lamina propria of small intestine; SPP, specific pathogen-free; Treg, regulatory T.

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Materials and Methods

Mice

Inbred C57BL/6J (B6) mice, CX3CR1-GFP (B6.129P-Cx3cr1tm1Li/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−/−/Oprtm1Mom) 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.

Colonic 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 1 mM DTT and 1 mM 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. no. C-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 x 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 NycosPrep (cat. no. 1002380; Axis-Shield, Oslo, Norway), overlaid with RPMI 1640 medium, and centrifuged at 4°C and 9,500 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-CD3ε mAb 145-2C11 (cat. no. 553064; BD Biosciences, Heidelberg, Germany), PE-conjugated anti-CD19 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⁺, CX3CR1⁺, and CX3CR1⁻ subsets were sorted using the FACSaria system (BD Biosciences).

CD45RBhigh CD4 T cell transfer colitis

CD T cell populations were isolated from the spleen of Foxp3-EGFP (B6, Cg-Foxp3m2tm1Ayj) 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; EbiScience, San Diego, CA) and a biotinylated mAb binding CD45RB 16A (cat. no. 553097; BD Biosciences), followed by the second-step reagent Streptavidin-Pacific-Chlorophyll-a Protein-Cy5.5 (cat. no. 551419; BD Biosciences). CD45RBhigh/Foxp3-EGFP⁺ CD4 T cells from the CD4⁺ population were enriched using the FACSaria system (BD Biosciences) to a purity >95%. Purified CD45RBhigh/Foxp3-EGFP⁺ CD4 T cells were injected i.p. (3 x 10⁶ cells/mouse) into congenic Rag-1⁻/⁻/CX3CR1⁻/⁻ mice. In some experiments, cell transfers were carried out with Foxp3-EGFP⁺ CD4 T cells or nonfractionated CD4 T cells containing CD45RBhigh and CD45RBlow 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 CD16/CD32 (0.5 μg/ml the Ab-PE); anti-FcγRII/III mouse mAb (BD Biosciences). The forward narrow angle light scatter was used as an additional parameter to facilitate the exclusion of dead cells and aggregated cell clumps. Data were analyzed using FCS Express V3 software.

Confocal microscopy

The ileum and the colon from SPF or (age- and sex-matched) GF CXCR1⁻/⁻ mice were opened by longitudinal incision and rinsed with PBS, as previously described (13, 21). Living tissues were imaged with an LSM 510 Meta scanning microscope. Three-dimensional reconstructions were performed on a Zeiss LSM 5 work station. Image analysis was carried out with the LSM image browser and Adobe Photoshop CS3.

Stimulation of cLP DCs with TLR agonists, by CD40 ligation, or with CX3CL1

to determine the cytokine response to TLR stimulation or CD40 ligation, the indicated DCs (5 x 10⁵ cells/well) were cultured for 1–5 d in 200-μl round-bottom microwells in RPMI 1640 medium supplemented with 5% FCS. For CD40 ligation, we used CD40L-expressing J558 transfectants (5 x 10⁴ cells/well) that were irradiated with 10,000 radiation-absorbed dose. The J558 BALB/c myeloma cells (ATCC TIB60) transfected with murine CD40L were provided by Dr. Mogens Clausen, Panum Institute, University of Copenhagen, Copenhagen, Denmark. In some experiments, cLP DCs were stimulated with 10 μg/ml LPS from Escherichia coli (cat. no. L4391; Sigma-Aldrich), 10 ng/ml oligodeoxynucleotide (ODN; MWG Biotech, Ebersberg, Germany), or 100 ng/ml recombinant soluble CX3CL1 (cat. no. 571-MF-025, R&D Systems, Minneapolis, MN). Supernatants were collected from cultures at the indicated time points for cytokine determination.

OT-II T cell differentiation assays

CD4 OT-II T cells were isolated from the spleen of OT-II mice by MACS (cat. no. 130-090-860; Miltenyi Biotec). Cells suspended in RPMI 1640 medium (supplemented with 5% FCS, 2 mM L-glutamine, and antibiotics) at a density of 1 x 10⁶/ml were pulsed for 2 h with 10 μg/ml the Aβ₃₃-₃₇ peptide IQSVAHHAAEINAGR (recognized by the
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. 55216) and biotinylated mAb XMG1.2 (cat. no. 55444) for IFN-γ, mAb JES6-1A12 (cat. no. 55442), biotinylated mAb JES6-5H4 (cat. no. 55446) for IL-2, mAb TC11-18H10 (cat. no. 555068) and biotinylated mAb TC11-8H4.1 (cat. no. 555067) 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 cell cultured. 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 Peyers’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), CX3CR1+ DCs, and CD103+ DCs in spleen, MLN, cLP, and siLP in wild-type and heterozygous Cx3cr1GFP/WT B6 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 CX3CR1+ cells made up 40–50% of the pDCs and DN (CD4−CD8−) DCs, >80% of the CD4 DCs, and <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 CD8+ and DN DCs from spleen and MLNs expressed CD103 (Fig. 1C), as previously reported (24, 25). Distinct CX3CR1 and CD103 expression by DCs raised the possibility that they are expressed by nonoverlapping DCs.

Purified colonic CD11c+ cells from Cx3cr1GFP/GFP mice were stained for CD103 to identify cLP CD11c+ cells that express CD103 and/or CX3CR1. Dot blot analyses 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+ CD11c+ and CX3CR1−CD11c− cLP DCs by gating on CD68+CX3CR1+ (Fig. 1F). CX3CR1+ CD11c+ and CX3CR1+CD103+ DCs showed high MHC class II; intermediate PD-L1; low CD40, CD80, and CD86; and no αν (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 Cx3cr1GFP/GFP 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/GFP mice, we observed 1.6 ± 0.4 transepithelial processes per villus, but only 0.4 ± 0.2 dendrites per villus in GF Cx3cr1GFP/GFP 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/GFP 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/GFP 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, and particularly in the MLNs, from GF mice (Fig. 2D). We isolated 62.1 × 10^6 CD11c+ cells from the siLP of one SPF mouse. In GF animals, the cell isolation yielded 43.7 × 10^6 CD11c+ cells per small intestine. We isolated 20.8 × 10^6 versus 9.7 × 10^6 CD4−CX3CR1− CD11c+ cells and 21.3 × 10^6 versus 9.9 × 10^6 CX3CR1−CD103− DCs in SPF and GF animals, respectively. In contrast, the numbers of CD103+ DCs were increased in GF animals compared with SPF animals (24.1 × 10^6 versus 10.4 × 10^6; p = 0.04) and CX3CR1−CD103− DCs (p = 0.03) were significantly decreased in the siLP of GF animals compared with SPF animals (Fig. 2E). The increase in CD103+ DCs in the siLP of GF animals was 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 ± 10^6 and 56.7 ± 10^6, respectively). CX3CR1−CD11c+ DCs and macrophages were reduced in GF animals (7.6 ± 10^6) compared with SPF animals (19.3 ± 10^6). Additionally, CX3CR1−CD103− DCs were reduced in GF animals (9.9 ± 10^6) compared with SPF animals (15.2 ± 10^6). Reduced numbers of CD103+ DCs were observed in the cLP of GF animals (18.2 ± 10^6) compared with SPF animals (22.3 ± 10^6) (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 animals compared with GF animals.
SPF Cx3cr1GFP/" 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+, whereas the 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.

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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 TLR-9 expression. Increased TLR-9 and -2 expression by CX3CR1+ DCs isolated from the cLP of SPF animals was observed compared with CD103+ DCs (Fig. 3). Significant differences in TLR-4 expression were not observed between CX3CR1+ and CD103+ DCs (Fig. 3). Therefore, CX3CR1+ and 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+, CD103+, and CX3CR1+CD103− DC subsets differ in their induced cytokine expression profile in response to stimulation with CpG-containing ODN or after CD40 ligation.
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 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+CD68−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 , and TLR-2 (C). 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 subspecifications.

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 CX3CR12CD103+ 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

FIGURE 5. CX3CR1+ DCs induce differentiation of IFN-γ- and IL-17-producing CD4 T cells. A, CD103+, CX3CR1+, or CD103−CX3CR1− DCs (1 × 10^6 cells/well) pulsed for 2 h with 10 μg/ml OVA323-339 ISQAVHAAHAEINEAGR peptide (recognized by the transgene-encoded TCR of OT-II mice) were cocultured with OT-II CD4 T cells (1 × 10^5 cells/well). Supernatants were collected at the indicated time points and analyzed by ELISA. The indicated cytokines were detected by conventional double-sandwich ELISAs. *Significant differences between cocultures with CX3CR1+ DCs versus cultures with CD103+ DCs. B, CD103+ CX3CR1+ or CD103−CX3CR1− peptide-pulsed cells (1 × 10^5 cells/well) were cocultured with OT-II CD4 T cells (1 × 10^5 cells/well) in the presence of 10 ng/ml ODN. Supernatants were collected and analyzed by ELISA for IL-17A. In the nonparametric Student t test, p < 0.05 was considered statistically significant.

FIGURE 6. CD103+ DCs induce the differentiation of Foxp3-expressing regulatory CD4 T cells. A, Peptide-pulsed CD103+ or CD103−CX3CR1− DCs or CX3CR1+ cLP DCs were cocultured with OT-II/RAG2−/−Foxp3− CD4 T cells for 5 d. Harvested T cells were stained for CD4, CD25, and Foxp3 and analyzed by FCM. Gated CD4+CD25+ cells are shown in dot blots. B, Percentage of Foxp3-expressing OT-II CD4 cells cocultured with the peptide-pulsed CX3CR1+, CX3CR1−CD103+, or CD103− subset. In the nonparametric Student t test, p < 0.05 was considered statistically significant.
with 10 ng/ml LPS and/or 100 ng/ml CX3CL1 for 48 h. Supernatants were harvested, and the concentration of TNF-α was determined by ELISA. Harvested supernatants from 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 was determined by ELISA. Harvested supernatants from 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 RAG2−/− mice.

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

CD4 CD45RBhigh Foxp3-EGFP+ T cells were adoptively transferred into (homozygous or heterozygous for Cx3cr1) RAG2−/−/CX3CR1-GFP animals. In a first set of experiments, we compared the induction of colitis by transfer of equal numbers of sorted CD45RBhigh CD4 T cells, nonfractionated CD4 T cells (containing CD45RBhigh and CD45RBlow cells), or sorted Foxp3-EGFP CD4 T cells. RAG2−/− hosts that received CD45RBhigh Foxp3-EGFP+ CD4 T cells developed a rapid colitis within 30 d. After transfer of nonfractionated CD4 T cells into RAG2−/− hosts, the development of colitis was attenuated compared with hosts that received CD45RBhigh Foxp3-EGFP+ CD4 T cells. RAG2−/− hosts that received CD45RBhigh 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 CD45RBhigh transfer colitis studies. We determined the distribution of DCs in the cLP and MLNs of (homozygous or heterozygous for Cx3cr1) RAG2−/−/CX3CR1-GFP hosts with transfer colitis. We analyzed the fraction (percentage of all CD11c+ cells) and absolute numbers (per tissue/ per mouse) of CD103+ and CX3CR1+CD103+ DCs. The local accumulation CX3CR1+ DCs and macrophages seems to support inflammatory immune responses in the cLP.

CD11c+ cell numbers were >3-fold reduced in MLNs from Cx3cr1GFP/+ hosts compared with Cx3cr1GFP/GFP hosts. CX3CR1+ DCs were <2-fold reduced, and CD103+ and CX3CR1+CD103− DCs were >2-fold reduced in homzygous CX3CR1-GFP animals compared to heterozygous animals with transfer colitis (Table I). Hence, CX3CR1 deficiency was associated with reduced numbers of DCs in the MLNs of RAG2−/− hosts with transfer colitis.

CD4 CD45RBhigh transfer colitis is suppressed in the absence of CX3CR1

The transfer of CD4 CD45RBhigh T cells into congenic SPF RAG2−/− and (age- and sex-matched) heterozygous RAG2−/−/CX3CR1-GFP hosts resulted in the progressive loss of body weight (Fig. 8A), histopathological signs of colitis (Fig. 8B, 8C), repopulation of the host with CD4 T cells (Fig. 9A, Table II), accumulation of IFN-γ cells in the inflamed cLP (Fig. 9A, Table II), and the appearance of increasing serum levels of IL-17 and IFN-γ (Fig. 9B, 9C). Differences in TNF-α serum levels were not observed (Fig. 9D). In contrast, the adoptive transfer of CD4 CD45RBhigh T cells into homozygous RAG2−/−/CX3CR1-GFP animals was associated with reduced body weight loss (Fig. 8A), reduced histopathological signs of colitis (Fig. 8B, 8C), and a reduced increase in serum IFN-γ and IL-17 (Fig. 9A, 9B). The manifestation of colitis was delayed/suppressed in CX3CR1-deficient hosts and was associated with reduced numbers of DCs in the MLNs and decreased numbers of Th1 and Th17 subsets in the cLP. The commensal-dependent accumulation of CX3CR1+ 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+, CX3CR1+, and CD103−CX3CR1− CD11c+ DCs. The local accumulation CX3CR1+ 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 Gr1lox− monocytes have been observed in the presence of macrophage-CSF receptors (35, 36). An increased death or an impaired recruitment of monocytes from the bone marrow to the spleen has been suggested as a 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.

### Table I. CD11c+ cells isolated from the MLNs of RAG−/−Cx3cr1GFP/GFP and RAG−/−Cx3cr1GFP/+ mice with colitis transplanted with congeneric CD45 RBhigh CD4 T cells

<table>
<thead>
<tr>
<th>Host/Cx3cr1</th>
<th>CD45RBhigh CD4 T Cell Transfer</th>
<th>Isolated CD11c+ Cells per MLN (× 10^4 ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAG−/−Cx3cr1GFP/GFP</td>
<td>–</td>
<td>26.7 ± 3.7</td>
</tr>
<tr>
<td>RAG−/−Cx3cr1GFP/+</td>
<td>+</td>
<td>67.7 ± 14.7</td>
</tr>
<tr>
<td>RAG−/−Cx3cr1GFP/GFP</td>
<td>–</td>
<td>22.7 ± 8.5</td>
</tr>
<tr>
<td>RAG−/−Cx3cr1GFP/GFP</td>
<td>+</td>
<td>24.7 ± 10.7</td>
</tr>
</tbody>
</table>

Mean ± SEM of four individual mice per group are shown. Bold type indicates significant differences in the number and the percentage of the indicated CD11c+ population between homozygous and heterozygous immunodeficient 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. – indicated groups did not receive CD45RBhigh cells; + indicated groups with transfer colitis. *Total CD11c+ cells isolated from the MLN of the indicated RAG−/− hosts and of hosts transplanted with 3 × 10^5 B6 CD45RBhigh Foxp3-EGFP+ CD4 T cells. **Total numbers of the isolated CD11c+ subset isolated from the indicated RAG−/− hosts.

**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.
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 RAG2/2 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).

**FIGURE 9.** The presence of CX3CR1 increases IL-17 and IFN-γ expression in mice with transfer colitis. A, cLP CD4 T cells were isolated from RAG2/2 and heterozygous and homozygous CX3CR1-GFP/RAG2/2 hosts transplanted with CD45RBhigh Foxp3-EGFP+ CD4 T cells from donor 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-γ, TNF-α or IL-10, and analyzed by FCM. The presence of IL-17 (B), IFN-γ (C), and TNF-α (D) in the serum of CD45RBhigh CD4 T cell-transplanted heterozygous and homozygous CX3CR1-GFP/RAG2/2 and RAG2/2 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 RAG2/2/Cx3CR1-GFP+ and RAG2/2/Cx3CR1-GFP+ mice with colitis transplanted with congenic CD45RBhigh CD4 T cells

<table>
<thead>
<tr>
<th>Transfers</th>
<th>Recovered CD4 T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total CD4 T cellsa</td>
</tr>
<tr>
<td>CD45RBhigh CD4 RAG2/2/Cx3CR1-GFP+</td>
<td>67 ± 14</td>
</tr>
<tr>
<td>CD45RBhigh CD4 RAG2/2/Cx3CR1-GFP+</td>
<td>166 ± 13</td>
</tr>
<tr>
<td>CD45RBhigh CD4 RAG2/2/Cx3CR1-GFP+</td>
<td>154 ± 23</td>
</tr>
</tbody>
</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.

aCD4 T cells recovered from the cLP of indicated RAG2/2/Cx3CR1-GFP+ hosts transplanted with 3 × 10⁸ B6 CD45RBhigh Foxp3-EGFP+ CD4 T cells.

bTotal 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 responses after stimulation with CpG-containing ODNs. CX3CL1 increased the cytokine release by LPS-stimulated cLP DCs and that preferentially support the differentiation of Th1/Th17 cells. The enteric flora (20), which is driven by commensal-derived ATP and/or the presence of specific microbiota (i.e., Bacteroidetes) in the intestine (49, 50). The CX3CL1+ DCs were identified as cells that preferentially support the differentiation of Th1/Th17 cells.


