CX₃CR1⁺ cells facilitate the activation of CD4 T cells in the colonic lamina propria during antigen-driven colitis

V Rossini¹, D Zhurina², K Radulovic¹, C Manta¹, P Walther³, CU Riedel² and JH Niess^{1,4}

Dendritic cells (DCs) and macrophages populate the intestinal lamina propria to initiate immune responses required for the maintenance of intestinal homeostasis. To investigate whether CX₃CR1⁺ phagocytes communicate with CD4 T cells during the development of transfer colitis, we established an antigen-driven colitis model induced by the adoptive transfer of DsRed OT-II cells in CX₃CR1^{GFP/+} × RAG^{-/-} recipients challenged with *Escherichia coli* expressing ovalbumin (OVA) fused to a cyan fluorescent protein (CFP). After colonization of CX₃CR1^{GFP/+} × RAG^{-/-} animals with red fluorescent *E. coli* pCherry-OVA, colonic CX₃CR1⁺ cells but not CD103⁺ DCs phagocytosed *E. coli* pCherry-OVA. Degraded bacterial-derived antigens are transported by CD103⁺ DCs to mesenteric lymph nodes (MLNs), where CD103⁺ DCs prime naive T cells. In RAG^{-/-} recipients reconstituted with OT II cells and gavaged with OVA-expressing *E. coli*, colonic CX₃CR1⁺ phagocytes are in close contact with CD4 T cells and presented bacterial-derived antigens to CD4 T cells to activate and expand effector T cells.

INTRODUCTION

Because the intestinal immune system is exposed to a vast array of harmless and potentially harmful antigens, intestinal immune responses need to be tightly controlled to prevent unlimited inflammation, as highlighted by the development of inflammatory bowel diseases, such as Crohn's disease or ulcerative colitis.^{1,2} Dendritic cells (DCs) and macrophages process intestinal antigens to initiate and maintain intestinal homeostasis.³

Several DC and macrophage populations have been described in the small and large intestinal lamina propria.³ The expression of the integrin α_E (CD103) and the fractalkine receptor CX₃CR1 allows distinguishing major myeloid cell populations within the small intestinal lamina propria and colonic lamina propria (cLP). CD11b⁻ and CD11b⁺ DCs express CD103. CX₃CR1⁺ cells can be further distinguished into F480⁺CD11c⁻ macrophages, F480⁺CD11c⁺ mononuclear phagocytes, and F4/80^{low}CD11c⁺ DCs. CD103⁺ DCs originate from common DC precursors under the control of FMS-like tyrosine kinase 3.^{4,5}

In contrast, monocytes give rise to CX₃CR1⁺ cells under the control of granulocyte-macrophage-colony-stimulating factor.^{4,5} CD103⁺ DCs instruct gut-imprinting abilities of CD4 and CD8 T cells and generate regulatory T cells under homeostatic conditions in a mechanism depending on the presence of retinoid acid.^{6,7} The majority of CX₃CR1⁺ F4/80^(high) macrophages produce interleukin-10 (IL-10) in a CX₃CR1-dependent manner in the steady state,⁸ whereas a small population of CX₃CR1⁺ F4/80^(low) DCs secrete IL-12, IL-23, and tumor necrosis factor- α and are able to activate T cells.^{9–11} CX₃CR1⁺ cells constitutively sample commensal and pathogenic microbes, such as *Salmonella typhimurium* and *Citrobacter rodentium*,^{12,13} and play an important role in host defense to pathogens. In addition, CX₃CR1⁺ cells take up circulatory antigens that enter the lamina propria by passing the fenestrated endothelium of capillaries and cross-present the antigens to CD8 T cells in the lamina propria,¹⁴ indicating that CX₃CR1⁺ cells play an important role in regulating immune responses in the lamina propria.

¹Department of Internal Medicine I, Ulm University, Ulm, Germany. ²Institute of Microbiology and Biotechnology, Ulm University, Ulm, Germany. ³Electron Microscopy Facility, Ulm University, Ulm, Germany and ⁴Department of Visceral Medicine and Surgery, Division of Gastroenterology, Inselspital, Bern, Switzerland. Correspondence: JH Niess (jan.niess@insel.ch)

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For the programming of T-cell responses, myeloid cells within the cLP and small intestinal lamina propria acquire intestinal antigens. Acquired antigens are processed and presented to T cells. Recent work has identified several pathways by which luminal antigens are uptaken by the mucosal immune system. Small intestinal CX₃CR1⁺ phagocytes and CD103⁺ DCs have extensions reaching into the intestinal lumen.^{12,15,16} Small soluble luminal antigens are delivered by goblet cells to CD103⁺ DCs.¹⁷ Villous M cells are scattered throughout the small intestine and transport luminal antigens to the lamina propria,¹⁸ whereas soluble antigens are able to pass the paracellular space between intestinal epithelial cells.¹⁹ M cells of the follicle-associated epithelium of Peyer's patches are major sites for the transportation of luminal antigens to DCs located in the dome regions of Peyer's patches.²⁰ In response to pathogens, CD11c⁺ DCs of the large intestine are able to extend processes into the intestinal lumen.²¹ Because the large intestine is densely populated by microorganisms that form the intestinal microflora, constituents of the intestinal microflora need to be surveyed by the mucosal immune system.²² Recognition of the constituents of the intestinal microflora seems to be a key event in this process.²³ Most studies that have unraveled pathways by which luminal antigens enter the host have been carried out in the small but not large intestine. Whether colonic CX₃CR1⁺ mononuclear cells communicate with CD4 T cells has remained controversial.²⁴

Given the high abundance of CX₃CR1⁺ DCs and macrophages in the lamina propria of the large intestine, we hypothesized that CX₃CR1⁺ phagocytes sample constituents of the intestinal microflora and interact with CD4 T cells. CX₃CR1⁺ phagocytes cross the basal membrane of the intestinal epithelium and extend processes between intestinal epithelial cells. Using transfer experiments we could show by *ex vivo* confocal imaging that CD4 T cells are located in close contact to CX₃CR1⁺ phagocytes. After adoptive transfer of DsRed⁺ CD4 T cells in CX₃CR1-GFP/RAG^{-/-} animals colonized with *Escherichia coli* pCherry-ovalbumin (OVA), confocal imaging provided evidence that colonic CX₃CR1⁺ phagocytes sample commensal bacteria and communicate with CD4 T cells.

RESULTS

CX₃CR1⁺ phagocytes cross the basal membrane in the proximal colon of B6 and RAG^{-/-} mice

CX₃CR1⁺ DCs and macrophages extend processes into the intestinal lumen in a mechanism partly dependent on CX₃CR1.^{12,25} These extensions have been mainly observed in the distal small intestine.²⁶ Only little information on the location of macrophages and DCs in the large intestine is available.^{16,21} Colonic tissues of CX₃CR1^{GFP/+} and CX₃CR1^{GFP/+} × RAG^{-/-} animals were scanned from the surface of intestinal epithelial layer to the submucosa and image series reconstructed with three-dimensional (3D) reconstructive confocal microscopy. The intestinal epithelium and the mucosa (stained with Alexa Fluor 350 agglutinin (blue))

surround intestinal crypts (**Figure 1b** and **d**). Beneath the intestinal epithelium, CX₃CR1⁺ phagocytes form networks located in the cLP around intestinal crypts (**Figure 1** and **Supplementary Movies S1** and **S2** online). In deeper sections, CX₃CR1⁺ phagocytes but not the intestinal epithelium and mucus were imaged (**Figure 1a,c,e,f**). Significant differences between heterozygous CX₃CR1^{GFP/+} animals and CX₃CR1^{GFP/+} animals on RAG^{-/-} background were not observed. The 3D reconstruction of confocal image series through colonic tissues demonstrates that CX₃CR1⁺ DCs and macrophages are located beneath the intestinal epithelium. CX₃CR1⁺ phagocytes cross the basal membrane reaching into the colonic epithelium (**Figure 1e–g**). The numbers of phagocytes entering into the intestinal epithelium were less as compared with the ileum (**Supplementary Figure S1** online). After infection with the enteric pathogen *C. rodentium*, the numbers of phagocytes reaching into the intestinal epithelium increased in the ileum but not the proximal colon (**Supplementary Figure S1**). Overall, these data showed that CX₃CR1⁺ phagocytes cross the basal membrane of the intestinal epithelium and are in part located between intestinal epithelial cells.

CD4 T cells are located close to CX₃CR1 phagocytes in mice with transfer colitis

Published evidence suggested that CX₃CR1^{high} cells produce IL-10, expand regulatory T cells in oral tolerance models, and suppress proliferation of T cells in transfer colitis models.⁸ CX₃CR1^{low}F4/80^{low} cells are able to activate T cells *in vitro* and induce the differentiation of T helper type 1 cell/T helper type 17 cell responses.^{9–11} To study the location/interactions of CD4 T cells and CX₃CR1⁺ DCs and macrophages in the cLP, we established an adoptive transfer system in which DsRed⁺ CD62L⁺ CD4 T cells were transferred into CX₃CR1^{GFP/+} × RAG^{-/-} animals. CD62L⁺ CD4 T cells were isolated from the spleen (SPL) of transgenic DsRed animals, in which a chicken β-actin promoter drives DsRed expression, and adoptively transferred into RAG^{-/-} recipients (**Supplementary Figure S2**). Significant differences in body weight loss, rise of IL-17A levels in sera, and histopathological colitis scores between recipients receiving B6 CD62L⁺ CD4 T cells or DsRed⁺ CD62L⁺ CD4 T cells were not observed. This means that the DsRed transgene does not affect the ability of CD62L⁺ CD4 T cells to induce colitis in RAG^{-/-} recipients (**Supplementary Figure S2**).

DsRed⁺ CD62L⁺ CD4 T cells were then adoptively transferred into RAG^{-/-} and heterozygous CX₃CR1^{GFP/+} × RAG^{-/-} recipients (**Figure 2a**). Both RAG^{-/-} and heterozygous CX₃CR1^{GFP/+} × RAG^{-/-} recipients developed colitis as indicated by body weight loss, an increase in IL-17A and interferon-γ (IFN-γ) sera concentrations, and histopathological signs of colitis (**Figure 2a–d**). At 7 days after adoptive transfer, only few DsRed⁺ CD4 T cells could be detected by flow cytometry. Highest numbers of CD4 T cells were first observed in the mesenteric lymph nodes (MLNs). At day 21 after transfer, the numbers of T cells in the cLP were higher than

Figure 1 CX₃CR1⁺ phagocytes cross the basal membrane of the intestinal epithelium. (a) Sections beneath the epithelial layer heterozygous CX₃CR1^{GFP/+} and (b) sections within the intestinal epithelial layer are shown from *ex vivo* confocal images of heterozygous CX₃CR1^{GFP/+} and heterozygous CX₃CR1^{GFP/+} × RAG^{-/-} animals stained with Alexa Fluor 350 agglutinin. Sections of heterozygous CX₃CR1^{GFP/+} × RAG^{-/-} animals (c) beneath the intestinal epithelium and (d) within the epithelium are presented. Original magnification × 40/1.30. The three-dimensional (3D) reconstruction of confocal images series through the proximal colon from the surface of the epithelial layer to the submucosa of (e) heterozygous CX₃CR1^{GFP/+} and (f) heterozygous CX₃CR1^{GFP/+} × RAG^{-/-} animals are presented. Intestinal epithelial cells were stained with Alexa Fluor 350 agglutinin. Arrows indicate processes of CX₃CR1⁺ phagocytes reaching into the epithelium. The experiment was repeated three times. Original magnification × 40/1.30. (g) Schematic depiction, where CX₃CR1⁺ phagocytes are localized in the proximal colon.

in the MLNs, suggesting that T cells are first primed in the MLNs before expanding in the cLP (**Supplementary Figure S3**). At 14 days after transfer, DsRed⁺ CD62L⁺ CD4 T cells formed cluster/aggregates that were surrounded by CX₃CR1⁺ phagocytes and dispersed throughout the cLP 21 days after transfer (**Figure 2e**). The 3D reconstructive confocal microscopy of the cLP at days 14 and 21 after T-cell transfer showed that CX₃CR1⁺ cells and CD4 T cells are located in close contact (**Figure 2f** and **Supplementary Movie S3**), suggesting that CX₃CR1⁺ cells and CD4 T cells may interact during the development of transfer colitis.

CX₃CR1⁺ phagocytes sample *E. coli* pCherry-OVA

To further investigate the role of CX₃CR1⁺ phagocytes in sampling constituents of the intestinal microflora, we constructed an *E. coli* pCherry-OVA strain that constitutively expressed the red fluorescent protein mCherry fused to chicken OVA protein (**Figure 3a,b** and **Supplementary Figure S4A**). The expression of mCherry and OVA by the transformed *E. coli* was confirmed by fluorescence microscopy and (**Figure 3b**) by western blotting (**Supplementary Figure S4A**). Colonic tissues of CX₃CR1^{GFP/+} animals gavaged with *E. coli* pCherry-OVA for 21 days were then analyzed by

Figure 2 DsRed⁺ CD4 T cells are located close to CX₃CR1⁺ phagocytes during transfer colitis. **(a)** The body weight was determined after transfer of DsRed⁺CD62L⁺ CD4 T cells in RAG^{-/-} and in heterozygous CX₃CR1^{GFP/+} × RAG^{-/-} recipients. Mean ± s.e.m. loss of body weight (%) per group is shown for the indicated groups. Seven animals were analyzed per group. In the nonparametric Student's *t*-test, *P* < 0.05 was considered statistically significant (**P* < 0.05). **(b)** interleukin-17A (IL-17A) and **(c)** interferon-γ (IFN-γ) serum concentrations from RAG^{-/-} or RAG^{-/-} and CX₃CR1^{GFP/+} × RAG^{-/-} mice transplanted with DsRed⁺ CD4 T cells is presented. Mean ± s.e.m. for each group is shown. This experiment was repeated twice. A *P*-value of < 0.05 in the two-tailed Student's *t*-test was considered statistically significant. **(d)** Histopathological scores of RAG^{-/-} and CX₃CR1^{GFP/+} × RAG^{-/-} mice transplanted with CD62L⁺ DsRed⁺ CD4 T cells. In the Student's *t*-test (nonparametric), *P* < 0.05 was considered statistically significant. **(e)** Large intestinal tissue samples were taken from all individual CX₃CR1^{GFP/+} × RAG^{-/-} animals transplanted with DsRed CD4 T cells at days 7, 14, and 21 after transfer, fixed in paraffin, and images were taken on a fluorescence microscope. Original magnification × 40. **(f)** After reconstitution of CX₃CR1^{GFP/+} × RAG^{-/-} mice with DsRed⁺ CD4 T cells, colonic tissues were analyzed by *ex vivo* confocal microscopy at days 7, 14, and 21 after cell transfer. The three-dimensional (3D) reconstructions of confocal image series (reaching from the surface of the intestinal epithelium to the submucosa) were carried out. The experiment was repeated four times. Original magnification × 40/1.30.

ex vivo confocal microscopy. mCherry-OVA *E. coli* could be detected within colonic crypts located close to intestinal epithelial cells (**Figure 3c**). CX₃CR1⁺ phagocytes have phagocytosed *E. coli* pCherry-OVA as shown by colocalization red fluorescent *E. coli* and green CX₃CR1⁺ phagocytes (**Figure 3c**).

To determine the relative numbers of internalized *E. coli* pCherry-OVA by defining CX₃CR1⁺ phagocytes, in which the red and green fluorescence intensity was determined by scatter blots. The analysis revealed that CX₃CR1⁺ cells have sampled 14.5 ± 1.4% of *E. coli* pCherry-OVA present in the colon, which

Figure 3 CX₃CR1⁺ phagocytes sample *Escherichia coli* pCherry-OVA. (a) Vector map of pCherry-OVA with relevant restriction sites and the gene encoding ovalbumin (OVA) and the bright red fluorescent protein mCherry. UTR, untranslated region. (b) Bright field and fluorescent microscopic images of *E. coli* DH10B, *E. coli* pAD₁-cCherry, and *E. coli* pCherry-OVA. (c) CX₃CR1^{GFP/+} mice were gavaged with 1×10^8 colony-forming units (CFUs) of *E. coli* pCherry-OVA for 3 days and analyzed by *ex vivo* confocal microscopy. Original magnification $\times 40/1.30$. (d) The percentage of internalized *E. coli* pCherry-OVA. The experiment was repeated twice.

is less than in the ileum (Figure 3d and Supplementary Figure S1C and D). This means that colonic CX₃CR1⁺ phagocytes take up constituents of the intestinal microflora in the colon.

CX₃CR1⁺ phagocytes stimulate CD4 T cells after colonization with *E. coli* pCherry-OVA

To identify the mononuclear phagocyte that sample *E. coli* from the intestinal lumen, CX₃CR1⁺ phagocytes and CD103⁺ DCs were isolated in the cLP from heterozygous CX₃CR1^{GFP/+} animals on B6 and on RAG^{-/-} background. Multicolor flow cytometry demonstrated that CX₃CR1⁺ phagocytes but not CD103⁺ DCs sampled *E. coli* pCherry-OVA from the colonic lumen during 3 weeks of feeding (Figure 4a.) The number of CX₃CR1⁺ cells containing *E. coli* pCherry-OVA increased, with highest numbers observed at day 21 (Figure 4b). CX₃CR1⁺ cells can be further distinguished into CD11c⁻ F4/80⁺, CD11c⁺ F4/80⁺, CD11c⁻ F4/80⁻, and CD11c⁺ F4/80⁻ cell populations (Figure 4c). Electron microscopy

demonstrated that F4/80⁺CD11c⁻ and F4/80⁺CD11c⁺ are characterized by phagocytic vacuoles and primary and secondary lysosomes that are typical morphological features of macrophages. In contrast, the F4/80⁻CD11c⁺ population has surface dendritic projection, lacks phagocytic vacuoles, and has less cytoplasm than the F4/80⁺ cells, which are typical features of DCs. The CD11c⁻F4/80⁻ population has a high nucleus/cytoplasm relation and could resemble natural killer cells in our cell preparations (Figure 4d). *E. coli* pCherry-OVA was mainly located in F4/80⁺CD11c⁻ and F4/80⁺CD11c⁺ phagocytes as these cells contained more red fluorescent *E. coli* as compared with F4/80⁻CD11c⁺, CD11c⁻F4/80⁻ cells, and CD103⁺ DCs (Figure 4e,f). Texas red dextran uptake assays confirmed that F4/80⁺CD11c⁻ and F4/80⁺CD11c⁺ cells have the highest phagocytic ability among the CX₃CR1⁺ cells (Supplementary Figure S5). To test the ability of phagocytes to activate CD4 T cells, sorted phagocyte populations from CX₃CR1^{GFP/+} animals gavaged for 3 weeks with *E. coli* pCherry-OVA were cocultured with OT-II cells for 3 days, and

IL-17A and IFN- γ production was determined by enzyme-linked immunosorbent assay (ELISA; **Figure 4g,h**). The F4/80[−]CD11c⁺ cell population isolated from the cLP of animals gavaged with *E. coli* pCherry-OVA had the highest ability to induce the secretion of IL-17A and IFN- γ by T cells. Overall, our data demonstrated that colonic CX₃CR1⁺ phagocytes sample *E. coli* from the intestinal lumen. F4/80⁺CD11c[−] and F4/80⁺CD11c⁺ cells are more efficient than F4/80[−]CD11c⁺ cells in sampling *E. coli* pCherry-OVA from the intestinal lumen. The F4/80[−]CD11c⁺ population has a high ability to stimulate CD4 T cells, indicating that CX₃CR1⁺ cells can uptake *E. coli* pCherry-OVA from the intestinal lumen and stimulate the immune responses of T cells.

CX₃CR1⁺ phagocytes interact with OVA-specific T cells during OVA-driven transfer colitis

Next, we constructed an *E. coli* pCFP-OVA that expresses cyan fluorescent protein (CFP) fused to OVA (**Figure 5a,b** and **Supplementary Figure S4A**). The CFP-OVA, as well as the mCherry-OVA, proteins produced by *E. coli* were able to induce an immune response of OVA-specific OT-II cells *in vitro*. ELISA analyses showed the increased production of IFN- γ by OT-II cells cocultured with CFP-OVA or mCherry-OVA producing *E. coli* when compared with OT-II cells stimulated with CFP or mCherry *E. coli* only (**Supplementary Figure S4B** and **C**).

DsRed animals were crossed with OT-II animals to obtain DsRed $\times$ OT-II transgenic mice (**Figure 5c**). This approach allows imaging of *E. coli* pCFP-OVA in CX₃CR1^{GFP/+} $\times$ RAG^{−/−} animals reconstituted with DsRed OT-II cells. DsRed⁺ CD62L⁺ OT-II T cells were adoptively transferred into CX₃CR1^{GFP/+} $\times$ RAG^{−/−} recipient animals. Reconstituted CX₃CR1^{GFP/+} $\times$ RAG^{−/−} recipients were gavaged every second day with *E. coli* pCFP-OVA, and the body weight was monitored. Reconstituted CX₃CR1^{GFP/+} $\times$ RAG^{−/−} recipients challenged with CFP-OVA⁺ *E. coli* lost body weight and developed clinical signs of colitis (**Figure 5d**). In part, the development of antigen-driven colitis depends on CX₃CR1, because an attenuated colitis was observed in CX₃CR1^{GFP/GFP} $\times$ RAG^{−/−} recipients (that lack CX₃CR1;

Supplementary Figure S6A–D). The *ex vivo* 3D reconstructive confocal imaging of colonic tissues was carried out on days 7, 14, and 21 after reconstitution of heterozygous CX₃CR1^{GFP/+} $\times$ RAG^{−/−} recipients with DsRed⁺ OT-II cells (**Figure 5e**). At 7 days after reconstitution, in heterozygous CX₃CR1^{GFP/+} $\times$ RAG^{−/−} recipients, only few OT-II cells were observed in the cLP in which few CX₃CR1⁺ phagocytes have sampled the CFP-OVA *E. coli*. Increasing numbers of DsRed⁺ OT-II cells could be observed 14 days after transfer of OT-II cells in RAG^{−/−} recipients. CX₃CR1⁺ phagocytes that have sampled *E. coli* pCFP-OVA are located close to DsRed⁺ OT-II cells. At 21 days after transfer, DsRed⁺ OT-II cells are dispersed throughout the cLP in close proximity to CX₃CR1⁺ phagocytes that have sampled CFP-OVA⁺ *E. coli* (**Figure 5e** and **Supplementary Movie S4**). At 21 days after cell transfer, DsRed⁺ OT-II cells were isolated from the cLP of reconstituted CX₃CR1^{GFP/+} $\times$ RAG^{−/−} recipients, stained for CD25, CD103, interferon-gamma receptor, and CD69, and analyzed by flow cytometry (**Figure 5f**). These cells displayed an activated phenotype as compared with OT-II cells from DsRed $\times$ OT-II transgenic donor mice as demonstrated by an increased expression of all the activation markers tested (**Figure 5f**). Hence, CX₃CR1⁺ phagocytes sample *E. coli* pCFP-OVA and are located close to CD4 T cells to communicate with T cells during the development of antigen-specific transfer colitis.

CD103⁺ DCs but not CX₃CR1⁺ phagocytes carry antigens derived from *E. coli* pCherry-OVA to the MLNs

Recent work indicated that the migration of CX₃CR1⁺ phagocytes to MLNs depends on the intestinal microflora.^{27,28} We analyzed the SPL, MLN, colonic tissues, and the cecum of CX₃CR1^{GFP/+} $\times$ RAG^{−/−} mice gavaged with 1×10^8 *E. coli* pCherry-OVA for the presence of *E. coli* pCherry-OVA by spotting tissue homogenates on agar plates in the presence of ampicillin. *E. coli* pCherry-OVA could only be cultured from homogenates of the colon and cecum but not of the MLNs and SPL (**Figure 6a**). Flow cytometry analyses of CX₃CR1⁺ phagocytes indicated that *E. coli* pCherry-OVA did not appear in the MLNs and SPLs of animals colonized for 3 weeks with *E. coli* pCherry-OVA (**Figure 6b**). *E. coli* pCherry-OVA could not be detected in the cLP or MLN CD103⁺ DCs as

Figure 4 F4/80[−]CX₃CR1⁺ cells have a higher ability in stimulating CD4 T cells than F4/80⁺CD11c⁺CX₃CR1⁺ cells. (a) CX₃CR1⁺ cells and CD103⁺ dendritic cells (DCs) were isolated from the colonic lamina propria (cLP) of heterozygous CX₃CR1^{GFP/+} animals gavaged with 1×10^8 colony-forming units (CFUs) of *Escherichia coli* pCherry-OVA for 7, 14, or 21 days, stained for CD11c and F4/80, and analyzed by multicolor flow cytometry. OVA, ovalbumin. Histograms were obtained by gating on CX₃CR1⁺ cells or on CD103⁺CD11c⁺ DCs. Numbers in histograms represent the percentage of positive cells (that have taken up *E. coli* pCherry-OVA). (b) The total numbers of CX₃CR1⁺ phagocytes that have sampled *E. coli* pCherry-OVA were calculated and presented as mean $\pm$ s.e.m. Seven animals were analyzed per group. In the nonparametric Student's *t*-test, a $P < 0.05$ was considered as statistically significant. (c) After gating on CX₃CR1⁺ cells F4/80 vs. CD11c dot blots were obtained. Numbers indicate the percentage of the respective population. (d) Electron microscopy of sorted indicated cell populations was carried out. (e) Histograms were obtained after staining of the indicated surface molecule by gating on the respective population. Gray histograms were obtained from non-fed CX₃CR1^{GFP/+} animals, and open histograms were obtained from animals gavaged with *E. coli* pCherry-OVA. The numbers in each histogram represent the percentage of cells that have sampled *E. coli* pCherry-OVA. (f) The total numbers of the indicated cell population (that contains *E. coli* pCherry-OVA) were calculated from 7 animals after 3 weeks of feeding with *E. coli* pCherry-OVA; in the Student's *t*-test (nonparametric), $P < 0.05$ was considered statistically significant. (g) The respective cell populations were isolated from the cLP of CX₃CR1^{GFP/+} $\times$ RAG^{−/−} animals gavaged for 3 weeks, cocultured with OT-II cells, and interleukin-17A (IL-17A) or (h) interferon- γ (IFN- γ) concentrations were measured by enzyme-linked immunosorbent assay (ELISA) and presented as mean $\pm$ s.e.m. In the nonparametric Student's *t*-test, $P < 0.05$ was considered as statistically significant.

Figure 5 CX₃CR1⁺ phagocytes interact with OT-II cells during antigen-driven transfer colitis. **(a)** Vector map of pCFP-OVA with relevant restriction sites and the gene encoding ovalbumin (OVA) and the bright cyan fluorescent protein (CFP). UTR, untranslated region. **(b)** Bright field and fluorescent microscopic images of *Escherichia coli* DH10B, *E. coli* pAD₁-cCFP, and *E. coli* pCFP-OVA. **(c)** DsRed⁺ OT-II cells were isolated from the spleens (SPLs) of DsRed-OT-II transgenic animals, stained for CD4, CD62L, and V β 5-1, and analyzed by flow cytometry. **(d)** Body weight was measured every fourth day of the respective animals in the presence or absence of *E. coli* pCFP-OVA. The numbers of animals per group are given in brackets. Mean ($\pm$ s.e.m.) body weight loss (%) is presented. In the Student's *t*-test (nonparametric), $P < 0.05$ was considered statistically significant. **(e)** Colonic tissue samples were examined by *ex vivo* three-dimensional (3D) reconstructive confocal imaging series at days 7, 14, and 21 after transfer of DsRed⁺ OT-II cells in CX₃CR1^{GFP/+} $\times$ RAG^{-/-} recipients. Original magnification $\times 40/1.30$. **(f)** CD4 T cells were isolated from the colonic lamina propria (cLP) of the indicated groups 21 days after cell transfer and analyzed by multicolor flow cytometry. Histograms were obtained by gating on DsRed⁺ CD4 T cells. The respective isotype controls are presented in gray histograms, and the open histograms show the respective surface staining. Numbers indicate the percentage of positive cells.

Figure 8 Antigen-driven transfer colitis can be induced in $Lta^{-/-} \times RAG^{-/-}$ animals. (a) The body weight of the indicated animals was determined every fourth day. The numbers of animals per group are given in brackets. The body weight loss is presented as mean $\pm$ s.e.m. loss of body weight (%). In the one-way analysis of variance (ANOVA; nonparametric) test, $P < 0.05$ was considered statistically significant. (b) The presence of interleukin-17A (IL-17A) and of interferon- γ (IFN- γ) in sera was determined 21 days after cell transfer by conventional double-sandwich enzyme-linked immunosorbent assay (ELISA). The data are presented as mean $\pm$ s.e.m. In the nonparametric Student's t -test, $P < 0.05$ was considered statistically significant. (c) Histopathological scores of animals with colitis. $P < 0.05$ was considered statistically significant in the nonparametric Student's t -test. (d) Colonic tissue samples were taken from all individual $Lta^{-/-} \times RAG^{-/-}$ animals transplanted with DsRed⁺ OT-II T cells on days 7, 14, and 21 after transfer, stained with Alexa Fluor 350 agglutinin, and fixed in paraffin. Images were taken on a fluorescence microscope. (e) CD4 T cells were isolated from the colonic lamina propria (cLP) of the indicated RAG^{-/-} animals, surface stained, and analyzed by multicolor flow cytometry. Histograms were obtained by gating on DsRed⁺ CD4 T cells 21 days after cell transfer. The respective isotype controls are presented in gray histograms, and the open histograms show the indicated surface staining. Numbers indicate the percentage of positive cells. The experiment was repeated twice.

proteins CFP or mCherry fused to OVA. CD103⁺ DCs pick up degraded antigens and deliver the bacterial-derived antigens to the MLNs, where naive T cells are primed by CD103⁺ DCs.

Primed T cells may home back to the lamina propria, where CX₃CR1⁺ phagocytes are able to activate effector CD4 T cells to induce local immune responses.

In our study CX₃CR1⁺ cells but not CD103⁺ DCs of the cLP sampled *E. coli*. In contrast to CX₃CR1⁺ cells, CD103⁺ DCs were able to induce T-cell responses in the MLNs. This means that CD103⁺ DCs likely pick up degraded bacterial fragments and transport this material to the MLNs, where CD103⁺ DC prime naive CD4 T cells.

The exact location of CD103⁺ DCs has not been determined yet in the colon, where CX₃CR1⁺ cells are a heterogeneous population.^{9,10,29,30} In the small intestine, CD103⁺ DCs are able to take up luminal pathogens.³¹ CX₃CR1⁺ F4/80⁺ CD11c⁺ and CX₃CR1⁺ F4/80⁺ CD11c⁻ cells are efficient cells in capturing luminal antigens but are poor inducers of T-cell immune responses. In contrast, CX₃CR1⁺ F4/80⁻ CD11c⁺ are much less efficient in sampling luminal antigens but are strong inducers of CD4 T-cell responses. Macrophages are very efficient in sampling antigens but have a poor ability to stimulate CD4 T cells.^{30,32,33} In contrast, DCs are less efficient in sampling antigens but have a high capacity in activating CD4 T cells.^{30,32,33}

CX₃CR1⁺ phagocytes isolated from the MLNs of gavaged animals were not able to prime naive T cells. The translocation of bacterial fragments by CX₃CR1⁺ phagocytes is prevented by the intestinal flora to protect the host from an uncontrolled activation of the immune system induced by the intestinal microflora.²⁸ The challenge of germfree mice with OVA-expressing *E. coli* could lead to different results. In our experiments the animals were gavaged with a nonpathogenic *E. coli*. Using pathogens as carriers for an OVA expression system could be of significance for the outcome of our experiments. The current study focused on experiments with a nonpathogenic *E. coli* as carrier for an OVA-expressing system in the presence of the intestinal microflora. There are likely multiple layers, by which an overwhelming immune response is prevented. The translocation of bacterial fragments to the MLNs by CD103⁺ DCs but not CX₃CR1⁺ phagocytes could be a possible way of how an uncontrolled immune response is prevented in the presence of the intestinal microflora.

To test whether the mucosal immune system has compensatory mechanism for the activation of T cells in the MLNs, OT-II cells were adoptively transferred in LT α ^{-/-} $\times$ RAG^{-/-} and LT β R^{-/-} $\times$ RAG^{-/-} lacking secondary lymphoid structures. Development of transfer colitis was observed in LT α ^{-/-} $\times$ RAG^{-/-} and LT β R^{-/-} $\times$ RAG^{-/-} hosts confirming previous reports.^{34,35} We used an antigen-driven colitis model,^{36,37} in which homeostatic T-cell proliferation will not contribute to the development of colitis.³⁸ Lymphotoxin (LT)-dependent pathways have also been implicated in M-cell differentiation, $\gamma\delta$ T-cell differentiation, and induction of immunoglobulin A production.^{39–41} The genetic depletion of constituents of the LT pathway leads to multiple defects. Expansion of T cells in reconstituted LT-deficient RAG^{-/-} animals was observed in the cLP. Transfer colitis can be induced in splenectomized LT-deficient RAG^{-/-}³⁴ but the bone marrow could still be a site, where T cells are primed by DCs.

Understanding the pathways by which immune responses are initiated in the intestinal mucosa are of importance for our

understanding of the development of inflammatory bowel disease. It seems that different subsets of myeloid cells in gastrointestinal tract have defined roles in both homeostatic and inflammatory conditions. CX₃CR1⁺ cells with high F4/80 expression may protect the host by uptaking and degrading products of the intestinal microflora. Bacterial fragments are picked up by CD103⁺ DCs and transported to the MLNs where CD103⁺ DCs prime naive CD4 T cells. F4/80⁻ CX₃CR1⁺ cells are efficient in inducing effector T-cell responses in the cLP. Further studies need to address whether this pathway may play a major role in the development of inflammation in patients with inflammatory bowel disease. The selective targeting of a particular DC and macrophage subset could be an interesting approach for the treatment of patients with inflammatory bowel disease.

METHODS

Mice. Inbred C57BL/6J (B6) mice, RAG^{-/-} (RAG^{tm1Mom}), heterozygous, and homozygous CX₃CR1-GFP $\times$ RAG^{-/-}, LT α ^{tm1Dch} $\times$ RAG^{-/-}, LT β R^{-/-} $\times$ RAG^{-/-}, DsRed mice (B6.Cg-Tg(CAG-DsRed*MST)1Nagy/J), DsRed $\times$ OT-II, and transgenic OT-II mice were bred and kept under specific pathogen-free conditions in the animal facility of the Ulm University (Ulm, Germany). Female and male mice were used at 6–12 weeks of age. All animal experiments were performed according to the guidelines of the local animal use and care committee and the National Animal Welfare Law.

Construction of *E. coli* DH10B pCherry-OVA and pCFP-OVA. In order to trace the bacteria expressing the OVA protein *in vivo*, the *E. coli* DH10B strains were generated carrying translational fusions of OVA to either blue (CFP) or red (mCherry) fluorescent protein. In both constructs, CFP or mCherry without stop codon were fused to the N-terminus of OVA separated by a stretch of six glycines. In order to generate the translational fusions, the full-size OVA gene was first amplified using Ova_SpeI_fw and Ova_ClaI_rev from the plasmid (pCI-OVA)⁴² and cloned into the SpeI/ClaI digested pBluescript vector (Fermentas, Vilnius, Lithuania) using the the SpeI and ClaI restriction sites of the primers and vector to yield pBluescript-OVA (Tables 1 and 2). To generate pCFP-OVA plasmid containing the CFP fused to OVA, the CFP-coding gene was amplified together with the strong constitutive promoter *PhyPer* using plasmid pAD₁-cCFP⁴³ as a template and primers CFP_SacII_fw and CFP_SpeI_rev. The 959-bp PCR product was further cloned into pBluescript-OVA using the SpeI and SacI restriction sites of the primers and vector. The same cloning strategy was applied to obtain the pCherry-OVA plasmid with the exception that pAD₁-cCherry plasmid⁴³ was used as a template to amplify the mCherry-coding gene and *PhyPer* promoter using the CFP_SacII_fw and mCherry_SpeI_rev primers. Correct assembly of the translational fusions was verified by sequencing. The plasmids were shown to stably replicate in *E. coli* DH10B strain in the absence of the antibiotic pressure for at least 50 generations and were hence considered suitable for *in vivo* applications. Expression of the fusion proteins CFP-OVA and mCherry-OVA was verified by western blotting using polyclonal anti-OVA-specific antibodies. Immunogenicity of the fusion proteins was further confirmed in the assays using splenocytes of OT-II mice as described below. Fluorescent microscopy confirmed the correct folding and fluorescence of CFP-OVA (excitation at 450 nm, emission at 480 nm) and mCherry-OVA (excitation at 587 nm, emission at 610 nm).

CD4⁺CD62L⁺ T-cell transfer colitis and antigen-driven colitis model. SPL cells were isolated from B6, DsRed, or DsRed $\times$ OT-II mice; CD4⁺CD62L⁺ T cells were purified and injected intraperitoneally (3×10^5 cells per mouse) into RAG^{-/-}, into heterozygous or

Table 1 Primers used in this study

Primer	Cleavage site	Sequence (3' → 5') ^a
CFP_SacII_fw	SacII	GATCGAC <u>CCGCGG</u> TTCTTGAAGACGAAAGGGCC
CFP_SpeI_rev	SpeI	GATCGAACTAGTACCACCACCACCACCACCTTTGTAGAGTTCATCCATGC
mCherry_SpeI_rev	SpeI	GATCGAACTAGTACCACCACCACCACCACCCTTGTACAGCTCGTC
Ova_SpeI_fw	SpeI	GACCAGACTAGTATGGAATTTTGTTTTGATGTATT
Ova-Clal_rev	Clal	GACCAGATCGATTAAGGGGAAACACATCTGCC

^aRestriction sites of the respective enzymes sites are underlined.

Table 2 Plasmids used in this study

Plasmid	Resistance	Description	Source
pCI-OVA	<i>AmpR</i>	Constitutively expressed OVA under the control of <i>CMV</i> enhancer/promoter	Schirmbeck <i>et al.</i> ⁴²
pAD ₁ -cCFP	<i>CmR</i>	Constitutively expressed CFP under the control of <i>PhyPer</i> promoter	Balestrino <i>et al.</i> ⁴³
pAD ₁ -cCherry	<i>CmR</i>	Constitutively expressed mCherry under the control of <i>PhyPer</i> promoter. Control plasmid to rule out the influence of mCherry alone on the immune response.	Balestrino <i>et al.</i> ⁴³
OVA-pBluescript		1,137 bp OVA gene cloned into <i>SpeI/Clal</i> digested pBluescript.	This study
pCFP	<i>AmpR</i>	959 bp fragment containing CFP with the 5'-UTR of <i>hly</i> gene under the control of <i>PhyPer</i> promoter cloned in the <i>SacII/SpeI</i> sites of the pBluescript vector. Control plasmid to rule out the influence of CFP alone on the immune response	This study
pCFP-OVA	<i>AmpR</i>	959 bp fragment containing CFP with the 5'-UTR of <i>hly</i> gene under the control of <i>PhyPer</i> promoter cloned in the <i>SacII/SpeI</i> sites of the OVA-pBluescript plasmid.	This study
pCherry-OVA	<i>AmpR</i>	732 bp fragment containing mCherry with the 5'-UTR of <i>hly</i> gene under the control of <i>PhyPer</i> promoter cloned in the <i>SacII/SpeI</i> sites of the OVA-pBluescript.	This study

CFP, cyan fluorescent protein; CMV, cytomegalovirus; OVA, ovalbumin; UTR, untranslated region.

into homozygous CX₃CR1-GFP × RAG^{-/-}, into LTα^{tm1Dch} × RAG^{-/-}, or into LtβR^{-/-} × RAG^{-/-} mice. Antigen-driven colitis was induced by injecting (intraperitoneally) purified SPL DsRed⁺ OT-II or OT-II T cells (3 × 10⁵ cells per mouse) into heterozygous or homozygous CX₃CR1-GFP × RAG^{-/-}, into LTα^{tm1Dch} × RAG^{-/-}, and into LtβR^{-/-} × RAG^{-/-} mice that were fed every second day for 3 weeks with 1 × 10⁸ colony-forming units (CFUs) of CFP-OVA⁺ *E. coli* DH10B mutants. 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 hematoxylin and eosin. Histology of the large intestine was categorized as normal (score 0); mild colitis (score 1), with few inflammatory cells in the cLP, stroma edema, and a slight reduction of goblet cells; moderate colitis (score 2), with an intense inflammatory infiltration of the lamina propria, 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.^{44,45}

CD4 T-cell isolation from cLP. CD4 T cells were isolated from the cLP of RAG^{-/-}, CX₃CR1^{GFP/+} × RAG^{-/-}, CX₃CR1^{GFP/GFP} × RAG^{-/-}, LTα^{tm1Dch} × RAG^{-/-}, and LtβR^{-/-} × RAG^{-/-} animals. Segments of colon were washed with phosphate-buffered saline (PBS) to remove debris and mucus. The epithelium was removed by incubation at 37 °C for 10–15 min under gentle shaking with 1 mM dithiothreitol (and 1 mM EDTA for colon tissue) in 25 ml PBS supplemented with 1% fetal calf serum. The remaining tissue was washed in PBS to remove residual epithelial cells, and the supernatants were discarded. Intestinal tissues were cut into 2 × 2-mm pieces and

digested by incubation with 0.25 mg ml⁻¹ collagenase type VIII from *Clostridium histolyticum* (cat. no. C-2139; Sigma-Aldrich, St Louis, MO) for 30–45 min at 37 °C in RPMI under shaking. Supernatants were collected, from which lamina propria lymphocytes were pelleted. The lamina propria lymphocytes were resuspended in RPMI medium containing 35% Percoll (density 1.124 g ml⁻¹; 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 35%/70% Percoll interface were collected and washed twice.

Monoclonal antibodies. The following reagents and monoclonal antibodies (mAbs) from eBioscience were used: fluorescein isothiocyanate (FITC)-conjugated mAb binding CD4 GK1.5 (cat. no. 11-0041-86), allophycocyanin (APC)-conjugated mAb binding CD4 GK1.5 (cat. no. 17-0041-83), phycoerythrin-cyanin-7 (PE-Cy7)-conjugated mAb binding F4/80 (cat. no. 25-4801), PE-conjugated mAb binding F4/80 (cat. no. 12-480f-80), PE-conjugated mAb binding CD8α (cat. no. 12-0081-82), APC-conjugated mAb binding CD11c (cat. no. 17-0114-82), biotinylated mAb binding CD25 (cat. no. 13-0251-81), biotinylated mAb binding CD69 (cat. no. 13-0691-81), and biotinylated mAb binding CD62L (cat. no. 13-0621-85). From BD Pharmingen (Heidelberg, Germany) the following reagents were used: FITC-conjugated mAb binding Vβ 5.1, 5.2 (cat. no. 553189), biotinylated mAb binding CD103 (M290) (cat. no. 557493), and biotinylated mAb binding interferon-gamma receptor (cat. no. 558771). As a second step, reagent PerCP-Cy5.5-conjugated streptavidin (cat. no. 45-4317-80; eBioscience, Frankfurt, Germany) was used.

Flow cytometry analyses. Cells were washed twice in PBS/0.3% w/v bovine serum albumin supplemented with 0.1% w/v sodium azide. Nonspecific binding of Abs to Fc receptors was blocked by preincubation of cells with mAb 2.4G2 directed against the FcγRIII/II

CD16/CD32 (0.5 ng mAb per 10^6 cells). Cells were washed and incubated with 0.5 ng per 10^6 cells of the relevant mAb for 20 min at 4 °C and washed again twice. In most experiments, cells were subsequently incubated with a second-step reagent for 20 min at 4 °C. Multicolor flow cytometry analyses were performed using a FACS-Calibur or a FACSaria flow cytometer system (BD Biosciences, Heidelberg, Germany). 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 (Los Angeles, CA).

Fluorescent and confocal microscopy. CX₃CR1^{GFP/+} animals or animals reconstituted with OT-II DsRed CD4⁺CD62L⁺ T cells were inoculated orally every second day for 3 weeks with 1×10^8 CFUs of CFP-OVA⁺ or mCherry-OVA⁺ *E. coli*. The colon from specific pathogen-free (age and sex matched) CX₃CR1^{GFP/+} animals was opened by longitudinal incision and rinsed with PBS. Living tissues were imaged with an LSM 710 Meta scanning microscope (Oberkochen, Germany). The 3D reconstructions were performed on a Zeiss LSM 7 workstation. Image analysis was carried out with the LSM image browser. After staining with 20 $\mu\text{g ml}^{-1}$ wheat germ agglutinin conjugated with Alexa Fluor 350 (Invitrogen Molecular Probes cat. no. W11263, Carlsbad, CA), intestinal tissues were fixed with cold paraformaldehyde (1% in PBS, pH 7.4) for 3 h and frozen in OCT (Sakura). Cryosections of 6–7 μm thickness were analyzed using fluorescence (Zeiss, Oberkochen, Germany; HBO 100) microscopy. ImageJ software version 1.43u (Imaging System, Carlsbad, CA) was used to analyze the images.

Transmission electron microscopy. For transmission electron microscopy, mononuclear phagocytes were fluorescence-activated cell sorted (FACS) from 10 CX₃CR1^{GFP/+} mice, resuspended in 50 μl PBS buffer, fixed with 2.5% glutaraldehyde, 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) overnight, washed with PBS, and contrasted with 2% aqueous osmium tetroxide for 1 h. Increasing 1-propanol concentration series (30, 50, 70, and 90%) were used to dehydrate the cells; the cells were contrasted with saturated alcoholic uranyl acetate solution for 30 min at 37 °C and embedded in Epon resin. The sections were cut using the Ultracut UCT ultramicrotome (Leica, Wetzlar, Germany) using a diamond knife (Diatome, Biel, Switzerland) and mounted on copper grids for transmission electron microscopy. Finally, samples were contrasted in 0.3% lead citrate for 1 min and then imaged at an acceleration voltage of 80 kV on a transmission electron microscope (EM10 Carl Zeiss, Oberkochen, Germany).

OVA detection by western blotting. Equal amount of protein samples per lane was loaded and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were further blotted onto the nitrocellulose membrane with the Bio-Rad blotting apparatus (Munich, Germany). Efficiency of the transfer was controlled as the prestained protein ladder was completely seen on the membrane and absent from the gel. The membrane was incubated with the 5% bovine serum albumin solution at room temperature overnight to prevent unspecific antibody binding. The membrane was further incubated with a rabbit anti-OVA polyclonal antibody (Abbiotec, San Diego, CA; cat. no. 250803, 1 mg ml^{−1}), diluted 1:400–1:700. Incubation was performed at room temperature for 1.5 h. Membranes were washed three times every 5 min in the universal buffer. The membrane was incubated with the secondary anti-rabbit in mouse (Sigma-Aldrich), coupled to the horseradish peroxidase at room temperature for 1.5 h. Membranes were washed three times for 5 min. Signal was detected on photofilms using a luminescence detection kit (Pierce, Rockford, IL).

***E. coli* pCherry-OVA load in SPL, MLNs, cecum, and colon.** CX₃CR1^{+/GFP} × RAG^{−/−} mice were fed for 21 days with mCherry-OVA⁺ *E. coli*. The organs were isolated aseptically from animals and the organ weight was measured. The organs were then homogenized in sterile PBS. Serial dilutions of the homogenates were spotted on LB

plates containing ampicillin (100 $\mu\text{g ml}^{-1}$) incubated at 37 °C in a humidified atmosphere for 18 h. Forming units were counted, and bacterial load was calculated as CFUs per g tissue.

Texas red dextran uptake assay. After isolation of the indicated CX₃CR1⁺ cell populations from the cLP, cells were centrifuged, re-suspended in RPMI media, and treated with Texas red dextran (Sigma, Munich, Germany) in a final concentration of 0.5 mg ml^{−1}. Cells were incubated at 37 °C for 1 h. As negative control, cells were incubated with Texas red dextran at 4 °C (negative control) and then both groups were incubated at 4 °C for an additional 30 min. Cells were washed four times with FACS buffer and analyzed with a FACSCalibur flow cytometer.

Assessment of the detection limit in our assays. Bacterial culture (50 ml) expressing mCherry-Ova protein was grown overnight. After centrifugation, the pellet was washed, resuspended in 2 ml of RPMI medium, and from this moment constantly kept on ice in order to prevent protein degradation. After disruption of the cells in a Ribolizer (Precellys 24, Bertin Technologies, Montigny-le Bretonneux, France), cell debris was spun down (15,000 g, 20 min, 4 °C) and the supernatants were sterile filtered and used as crude bacterial extracts. Total protein concentration in crude extracts was determined by bicinchoninic acid assay (Pierce), according to the manufacturer's instructions (Thermo Scientific cat. no. 23227, Rockford, IL), and the presence of the red fluorescent protein was confirmed by absorbance measurement in Tecan microplate reader (Tecan, Wetzlar, Germany). The amount of mCherry-Ova was determined by densitometry analysis using ImageJ software. For this purpose the intensity of the band corresponding to mCherry-Ova (~68.3 kDa) was quantified relative to the band intensities of all proteins detected present on a Coomassie-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis. According to this quantification, mCherry-Ova protein represents 1.6% of all proteins. 1×10^5 CX₃CR1⁺ cells isolated from CX₃CR1^{GFP/+} mice were resuspended in RPMI media and titrated with crude extracts containing mCherry-OVA in a concentration of 0.1–2.5 mg ml^{−1} total protein. Cells were incubated at 37 °C for 1 h. As negative control, cells were incubated at 4 °C for 1 h. Then, both groups were incubated at 4 °C for additional 30 min. Cells were washed four times with FACS buffer and analyzed with a FACS Calibur flow cytometer.

Activation of OT-II cells. After inoculation of 1×10^8 CFUs CFP-OVA⁺ *E. coli* or of mCherry-OVA⁺ *E. coli* in CX₃CR1^{GFP/+} for 21 days, CX₃CR1⁺ cells were enriched from the colonic and small intestinal lamina propria of CX₃CR1^{GFP/+} mice using the FACSaria system (BD Biosciences) to a purity of 95%. Purified CX₃CR1⁺ cells were cocultured with OT-II cells from the SPL of OT-II mice (in the ratio of 1:1, we used 10^5 cells per wells) for 48 h in 96 round-bottom wells. IL-17A and IFN- γ concentration in supernatants were measured by ELISA. When OT-II cells were *in vitro* stimulated with CFP-OVA⁺ *E. coli* or mCherry-OVA⁺ *E. coli*, bacterial cells were inactivated with 5 $\mu\text{g ml}^{-1}$ of gentamicin after 1 h of incubation. OT-II cells isolated from SPLs (1×10^6 per well) cocultured with indicated dilutions of both bacterial cells (from 1×10^5 to 1×10^2 CFUs) for 48 h in 96-well round-bottom plates. As negative controls served cultures, in which CFP⁺ *E. coli* or mCherry⁺ *E. coli* (that did not express OVA) was added. ELISA was used to measure IFN- γ in the supernatants.

Cytokine detection by ELISA. Cytokines in supernatants and blood serums 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 XMGI.2 (cat. no. 554410) for IFN- γ , mAb TC11-18H10 (cat. no. 555068), and biotinylated mAb TC11-8H4.1 (cat. no. 555067) for IL-17A. Extinction was measured at 405/490 nm on a TECAN microplate-ELISA reader using EasyWin software (both from Tecan).

Statistics. A *t*-test for two unequal variances was used. $P < 0.05$ was considered statistically significant. A nonparametric one-way analysis of variance test was used for comparing more than two variances.

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

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DISCLOSURE

The authors declared no conflict of interest.

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