Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions

Olga Schulz, Elin Jaensson, Emma K. Persson, Xiaosun Liu, Tim Worbs, William W. Agace, and Oliver Pabst

Chemokine receptor CX3CR1+ dendritic cells (DCs) have been suggested to sample intestinal antigens by extending transepithelial dendrites into the gut lumen. Other studies identified CD103+ DCs in the mucosa, which, through their ability to synthesize retinoic acid (RA), appear to be capable of generating typical signatures of intestinal adaptive immune responses. We report that CD103 and CX3CR1 phenotypically and functionally characterize distinct subsets of lamina propria cells. In contrast to CD103+ DC, CX3CR1+ cells represent a nonmigratory gut–resident population with slow turnover rates and poor responses to FLT-3L and granulocyte/macrophage colony-stimulating factor. Direct visualization of cells in lymph vessels and flow cytometry of mouse intestinal lymph revealed that CD103+ DCs, but not CX3CR1-expressing cells, migrate into the gut draining mesenteric lymph nodes (LNs) under steady-state and inflammatory conditions. Moreover, CX3CR1+ cells displayed poor T cell stimulatory capacity in vitro and in vivo after direct injection of cells into intestinal lymphatics and appeared to be less efficient at generating RA compared with CD103+ DC. These findings indicate that selectively CD103+ DCs serve classical DC functions and initiate adaptive immune responses in local LNs, whereas CX3CR1+ populations might modulate immune responses directly in the mucosa and serve as first line barrier against invading enteropathogens.

The intestinal mucosa contains large numbers of mononuclear cells, including macrophages and DCs, which together are believed to play a central role in regulating mucosal innate and adaptive immune responses in both the steady-state and inflammatory setting (Kelsall, 2008). Expression of CD11c and CD11b has been used to distinguish DC subsets from macrophages in the intestine. However, separating subsets of mononuclear cells based on CD11c is complicated because this marker is also expressed on tissue macrophages (Hume, 2008). Furthermore, separating subsets of mononuclear cells based on CD11c is complicated because this marker is also expressed on tissue macrophages (Hume, 2008). Furthermore, there is currently little evidence to suggest that subdividing mononuclear lamina propria (LP) cells (LPCs) based on CD11c and CD11b defines populations with distinct turnover rates, origins, and functional properties.

Based on the observation that DC can penetrate epithelial monolayers in vitro and a close association of CD11c-expressing cells with the gut epithelium in vivo, Rescigno et al. (2001) put forward the idea of epithelial-associated DC that is capable of capturing luminal bacteria and transporting them into the intestinal LP. Further studies using transgenic mice expressing GFP under the control of the CX3CR1-promoter (CX3CR1+/GFP mice) confirmed that a major subset of mononuclear cells in the intestinal LP are capable of extending processes across the epithelial layer into the intestinal lumen (Niess et al., 2005). In vivo invasion-defective Salmonella enterica were associated with CX3CR1-expressing cells in the intestinal LP of transepithelial-proficient, but not transepithelial dendrite-deficient, CX3CR1GFP/GFP mice, suggesting that transepithelial dendrites play an
important role in sampling and uptake of luminal microbes (Niess et al., 2005; Hapfelmeier et al., 2008). Such transepithelial dendrites have also been observed in CD11c and MHCII reporter mice (Niess et al., 2005; Chieppa et al., 2006) even though their frequency and distribution remains controversial (Chieppa et al., 2006; Vallon-Eberhard et al., 2006). Together, these studies have led to the prevailing assumption that epithelial-associated CX3CR1+ mononuclear LPCs are DCs that play an important role in the initiation of intestinal adaptive immune response (Ressigno, 2003; Chieppa et al., 2006; Niess and Reinecker, 2006). Nevertheless, definitive evidence that CX3CR1+ LPCs are tissue DCs that are capable of migrating to draining mesenteric LN (MLN) and priming T cell responses has not been provided.

We, and others, have identified a subset of DC in the intestinal LP and MLN which expresses the integrin α chain CD103. These cells display an intrinsic ability to induce the gut-homing receptors CCR9 and α4β7-integrin on responding T cells (Annacker et al., 2005; Johansson-Lindbom et al., 2005) and FoxP3 Treg differentiation in vitro (Coombes et al., 2005) and FoxP3 Treg differentiation in vitro (Coombes et al., 2005; Johansson-Lindbom et al., 2005; Sun et al., 2007). Both of these functions appear to be caused by an enhanced ability of intestinal CD103+ DC to induce retinoic acid (RA) receptor signaling in responding T cells (Jaensson et al., 2008; Svensson et al., 2008) and are associated with higher expression of the gene encoding RALDH2 (retinaldehyde dehydrogenase 2), aldh1a2 (Coombes et al., 2007), a key enzyme involved in retinal metabolism. Importantly, CD103+ DCs are also present in human MLN and selectively induce RA receptor-dependent CCR9 expression on allogenic T cells (Jaensson et al., 2008). Thus, expression of CD103 identifies a DC subset that, under the influence of environmental conditioning in the gut and in the MLN (Agace, 2008; Hammerschmidt et al., 2008), confers typical properties of intestinal immune responses. CD103+ DCs are dramatically reduced in the MLN, but not LP, of CCR7-/− mice (Johansson-Lindbom et al., 2005; Worbs et al., 2006) and, in BrDU pulse-chase experiments, have been shown to turn over rapidly in the LP in the steady state and with delayed kinetics in the MLN, suggesting that CD103+ MLN DCs represent LP-derived migratory DCs (Jaensson et al., 2008). Finally, CD103+, but not CD103−, MLN DCs are capable of priming OT-I and OT-II cells ex vivo after oral administration of OVA, indicating that these cells play a central role in initiating T cell responses to soluble luminal antigen (Coombes et al., 2007; Jaensson et al., 2008). Despite these findings, it remains unclear whether intestinal CD103+ DC and CX3CR1+ cells represent phenotypically and functionally overlapping or distinct populations of cells in the intestinal LP.

In this paper, we demonstrate that CD103 and CX3CR1 expression defines distinct LP populations. Although CD103+ DCs are short lived, travel via intestinal lymph to the gut draining MLN, and efficiently present antigens to naive T cells, CX3CR1+ cells are comparably longer lived LP-resident cells, cannot be observed in intestinal lymph, and are poor at presenting antigen to naive T cells. Together, these results suggest a division of labor between gut-resident CX3CR1+ LPC and migratory antigen-presenting CD103+ DC in the intestinal immune system.

RESULTS

CX3CR1 and CD103 identify nonoverlapping subsets of LP mononuclear cells

Defining LP mononuclear cell subsets based on MHCII+CD11b, and CD11c is complicated because of variations in the expression levels of these markers, resulting in often overlapping and uncertain definitions of cell populations (unpublished data; Chieppa et al., 2006; Uematsu et al., 2006, 2008). Because a prominent subset of LPC, which is capable of extending cellular processes through the gut epithelium, has been described using CX3CR1+/GFP mice (Niess et al., 2005; Vallon-Eberhard et al., 2006), we used CX3CR1+/GFP mice to assess CX3CR1, MHCII, CD11c, and CD103 expression on LPC by confocal microscopy and flow cytometry. Consistent with a putative function in antigen sampling, CX3CR1+/GFP+ cells localized close to the intestinal epithelium (Fig. 1 A). In confocal microscopy, CX3CR1/GFP+ LPC showed variable expression of CD11c and no expression of CD103 (Fig. 1 A). In contrast, CD103 was evident as CD11cintCD103+ LPC with no detectable expression of CX3CR1/GFP (Fig. 1 A) and these cells preferentially localized in the villus core (Fig. 1 A). Enumeration of CX3CR1/GFP+ and CD103+ CD11c+ LPC revealed roughly three- to fourfold more cells of the former compared with the latter LP population (Fig. 1 A). These observations suggest that CD103 and CX3CR1/GFP identify nonoverlapping subtypes of small intestinal LPC.

Flow cytometry of isolated LPC confirmed these observations. The CD103+ LPC population comprised a substantial fraction of MHCII+CD11c+ cells that showed very weak CX3CR1/GFP signals (Fig. 1 B and Fig. S1 A). LPC expressing CX3CR1/GFP levels that potentially might be detected by fluorescent microscopy could be divided into two distinct populations distinguished by high and intermediate CX3CR1/GFP expression (Fig. 1 B). Freshly isolated CX3CR1/GFPhigh LPCs were MHCII−CD11b+. These cells also expressed the macrophage markers CD14 and F4/80 and most expressed CD11c, although at consistently lower levels than CD103+ MHCII+ cells. Based on these markers, CX3CR1/GFPint cells appear to represent a homogenous LPC population (Fig. 1 B and Fig. S1 B). Although the majority of CX3CR1/GFPint LPC also expressed MHCII and CD11b, this population appeared phenotypically more heterogeneous. Although most CX3CR1/GFPint LPC expressed F4/80 and CD14, albeit at lower levels than CX3CR1/GFPhigh cells, this population also contained a variable proportion of MHCII+ cells that were predominantly GR-1+CD11b+CD11c+CCR2high, a phenotype which is similar to that of circulating inflammatory monocytes (Fig. 1 B, Fig. S1 C, and not depicted; Landsman et al., 2007). For brevity, we refer to CD103+MHCII+CD11c−CX3CR1− LPC as CD103+ DC. Because CX3CR1/GFP-expressing LPCs share phenotypic traits with both macrophages...
Figure 1. **CX3CR1 and CD103 characterize distinct sets of LP mononuclear cells.** (A) 50-µm cryosections of CX3CR1/GFP mice were stained for CD11c and CD103 and analyzed by confocal microscopy. The image shows an orthogonal projection of a 7.5-µm z-stack depicting CX3CR1/GFP (green), CD103 (red), and CD11c (blue). The boxed region is shown in detail in split channels. The dashed lines indicate the epithelial border/basal lamina. Bar, 10 µm. The localization of CX3CR1/GFP+ and CD103+CD11c+ cells in the proximal ileum with respect to the basal epithelium and their numbers were determined using 7-µm-thick cryosections documented by epifluorescence microscopy. Diagrams depict mean and 25th and 75th percentiles as boxes. Sections obtained from four mice in two independent experiments were evaluated and a total of 116 CD103+ and 584 CX3CR1+ cells were analyzed. ***, P < 0.001.

(B) Flow cytometric analysis of CX3CR1+/GFP LPCs. CD103+ DCs express very low levels of CX3CR1 and were identified by subgating the CD103+/CX3CR1low/low population on CD11c+MHCII+ cells. CD11clow/MHCII− cells in this plot are removed when gating on CD3−CD19− cells, suggesting they are T cells (not depicted). CX3CR1+ LPCs could be further subgrouped into CX3CR1int and CX3CR1high LPC populations. All three populations were analyzed for expression of CD11b and F4/80. Numbers indicate mean percentage (SD) of cells in the respective gate of 10 mice analyzed in at least four independent experiments. The total percentage of CD103+ DC among CD45+ cells is 1.9% (SD, 1).
represented CX3CR1+ LPC. To assess this possibility, a small intestinal fragment, including the draining MLN, was isolated from CX3CR1/GFP mice and transplanted into syngenic WT recipients. 9 d after transplantation, the frequency of CX3CR1+ LPC in the grafted intestinal fragment was determined by immunofluorescence microscopy and compared with non-transplanted CX3CR1+/GFP mice. Strikingly, and in marked contrast to CD103+ DCs (Jaensson et al., 2008), CX3CR1+ LPCs were not reduced in the grafted small intestine compared with unmanipulated CX3CR1+/GFP mice (Fig. 2 B). This indicates that CX3CR1+ LPCs are not rapidly replaced from extraintestinal sources, even though surgery might promote loss of cells from the transplanted tissue. To compare the turnover of CX3CR1+ LPC and CD103+ DC in the steady state, CX3CR1+/GFP mice were injected with a single dose of BrdU. Consistent with our observation that these cells turn over rapidly in vivo (Jaensson et al., 2008), 9 h after injection almost 10% of CD103+ DC had incorporated BrdU (Fig. 2 C). In contrast, both CX3CR1int and CX3CR1high LPCs showed significantly lower rates of BrdU incorporation (Fig. 2 C). Finally, to determine the impact of different DC/myeloid cell growth factors on CX3CR1+ LPC and CD103+ DC compartments in vivo, CX3CR1+/GFP mice were injected with melanoma cells constitutively secreting Flt3L or GM-CSF and, after 6–12 d, the numbers of CX3CR1+ LPCs and CD103+ DCs were determined by fluorescence microscopy and flow cytometry. CD103+ DCs efficiently expanded in mice carrying either Flt3L (Fig. 3, A and B) or GM-CSF–producing tumors (Fig. 3 B). In contrast, CX3CR1high LPC failed to respond to either cytokine, whereas CX3CR1int LPC showed expansion to Flt3L. Collectively, these results demonstrate that CD103+ DC and CX3CR1+ LPC display different turnover rates and responses to Flt3L and GM-CSF and, thus, likely derive from distinct precursor populations.

**Figure 2.** CX3CR1+ LPCs, but not CD103+ DCs, display long-term persistence in small bowel transplants. (A) CD45.2+ intestinal fragments were grafted into CD45.1+ recipients and analyzed by fluorescence microscopy. 6 d after transplantation, the majority of CD11c+MHCII+ (arrows) cells in the grafted tissue did not express CD45.2, i.e., had been replaced by host cells. In contrast, CD11c+ cells with low MHCII signal (arrowheads) were mostly CD45.2+; which is indicative of donor origin and long-term persistence in the grafted tissue. Bars, 50 µm. Six mice were analyzed in six experiments. (B) Intestinal fragments were grafted from CX3CR1+/GFP donors into WT recipients. 9 d after transplantation, the number of CX3CR1/GFP+ cells in the grafted tissue was enumerated by epifluorescence microscopy and compared with unmanipulated CX3CR1+/GFP mice (control). Error bars represent SD. Bars, 20 µm. Two mice were analyzed in two experiments. (C) CX3CR1+/GFP mice were injected i.p. with 2 mg BrdU and, 9 h later, the percentage of BrdU-positive CD103+ DC and CX3CR1+ LPC was determined by flow cytometry. Six mice were analyzed in three independent experiments. Error bars represent SEM. Results were obtained with a Wilcoxon signed rank test. * , P < 0.05.
CD103+ DCs, but not CX3CR1+ LPCs, migrate in intestinal lymph

A paradigmatic view of the DC life cycle is that immature DCs sample antigen in the periphery, mature, and migrate via the lymphatics into the draining LNs to present antigen. We have previously provided indirect evidence that CD103+ DCs are a migratory population of cells that continually migrate to the draining MLN (Johansson-Lindbom et al., 2005; Hammerschmidt et al., 2008; Jaensson et al., 2008). To directly determine whether CD11c+ DCs and/or CX3CR1+ LPCs migrate from the small intestine to the MLN, we performed multiphoton confocal microscopy on

Figure 3. CX3CR1+ LPC and CD103+ DC display different responsiveness to Flt3L and GM-CSF in vivo. Melanoma cells secreting Flt3L or GM-CSF were injected subcutaneously into CX3CR1+GFP mice and animals were sacrificed 6–12 d later. (A) 12 d after injection of Flt3L-secreting tumor cells, confocal microscopy revealed massive expansion of intestinal CD103+ DC but not CX3CR1+ LPC. Images show representative stainings for CX3CR1/GFP (green), CD103 (red), and CD11c (blue). The boxed region in split channels is shown on the bottom. Arrowheads indicate CD11c+CD103+ LP DC and arrows mark CD11c−CD103+ cells, most likely representing T cells. Three mice were analyzed in three independent experiments. Bars, 20 µm. (B) The total number of isolated CD103+ DC, CX3CR1int LPC, and CX3CR1high LPC in tumor-bearing mice was determined by flow cytometry. Mean (SD) is shown of at least six mice per group analyzed in two (GM-CSF) and three (Flt3L) independent experiments. Results were obtained with an unpaired Student’s t test. **, P < 0.01; ***, P < 0.001.
mesenteric lymph vessels. Lymph vessels were readily identified in olive oil–fed mice as whitish vessels running in parallel to mesenteric blood vessels (Fig. 4 A; Hammerschmidt et al., 2008). To facilitate identification of lymph vessels by fluorescence microscopy, we injected fluorescently labeled dextran 30 min before sacrificing the mice. Fluorescently labeled dextran selectively highlights blood vessels shortly after i.v. injection, whereas 30 min after injection, blood and lymph vessels are both labeled (Fig. 4 A). Lymph vessels were readily visible by bright-field microscopy, TRITC fluorescence signal, and a second harmonics signal, visualizing the collagen-rich vessel wall. Cells present in the vessel lumen appeared as dark TRITC-excluding shadows (Fig. 4 B). Using mice expressing enhanced YFP (EYFP) under control of the CD11c promoter (CD11c-EYFP mice), we readily identified CD11c-expressing cells in intestinal lymph

![Figure 4](https://jem.rupress.org/)(A) Visualization of MLN-afferent lymphatics. 1 h after gavage of 200 µl of olive oil, lymphatics appear as bright/white vessels running in parallel to mesenteric blood vessels (left). FITC- and TRITC-labeled dextran injected i.v. 2 and 30 min before analysis (green and red, respectively) can differentially highlight blood vessels or both blood vessels and lymphatics. In the merge (right) image, blood vessels appear yellow and lymph vessels red. Bar, 1 mm. (B) Two-photon microscopy of MLN-afferent lymphatics of CD11c-EYFP and CX3CR1+/GFP mice in the steady state or 5 h after oral administration of R848. TRITC-labeled dextran was injected i.v. 30 min before mice were sacrificed to visualize the vessel lumen (red). Collagen-rich vessel walls display a second harmonic signal (blue). The images show orthogonal projections of 20-µm-thick (CD11c-EYFP mice) or 5-µm-thick (CX3CR1+/GFP mice) volumes. Bars, 50 µm. CD11c-positive cells were readily detectable in steady-state lymphatics (top left, green) and in increased numbers 5 h after gavage of 10 µg R848 (bottom left). CX3CR1-positive cells typically occurred in the shape of stellate morphology cells on the outside of vessel tubes or within the vessel wall (top right). Cells not expressing the reporter GFP/YFP appeared as TRITC-excluding shadows in the lumen. Pictures shown are from non–Peyer’s Patch draining lymphatics. The following numbers of independent experiments were each performed with single mice: CD11c-EYFP, five; CD11c-EYFP+R848, two; CX3CR1+/GFP, four; and CX3CR1+/GFP+R848, five.
vessels (Fig. 4 B, top left). CD11c/YFP+ cells in lymph vessels showed a rounded morphology, and careful analysis of Z-stacks confirmed intraluminal localization as judged by the collagen/SHG signal. CD11c/EYFP-expressing cells were scarce but could be demonstrated in almost all image stacks analyzed (unpublished data). Extending these experiments to CX3CR1+/GFP mice, CX3CR1/GFP-expressing cells were detectable in surrounding fatty tissue, either as scattered cells or in compact cell clusters (not depicted), and occasionally on the extraluminal side of the lymph vessels (Fig. 4 B, top right; and Video 1). This latter population displayed stellate cell morphology and in some cases extended protrusions into the lymph vessel. However, in striking contrast to CD11c/EYFP+ cells, CX3CR1/GFP+ cells were almost undetectable within intestinal lymph vessels (Video 1). Of four mice analyzed, only in one case were a total of four CX3CR1/GFP-expressing cells observed in the lymph vessel lumen.

R848 signaling via Toll-like receptor (TLR) 7/8 enhances the migration of DC from the LP into the MLN (Yrlid et al., 2006). Such R848-induced mobilization of DC is accompanied by a massive increase in serum levels of proinflammatory cytokines and does not depend on direct TLR stimulation on the DC (Yrlid et al., 2006). We thus used oral R848 application to investigate the presence of CD11c+ and CX3CR1-expressing cells in intestinal lymph under inflammatory conditions. 5 h after oral administration of R848 to CD11c-EYFP mice, the number of CD11c/EYFP+ cells in intestinal lymph was markedly increased compared with steady-state conditions (Fig. 4 B, bottom left; and Video 2). Oral application of R848 to CX3CR1+/GFP mice, however, failed to mobilize CX3CR1+ cells into intestinal lymph (Fig. 4 B, bottom right). Thus, direct visualization of CD11c- and CX3CR1-expressing cells in intestinal lymph revealed that CD11c+, but not CX3CR1+, cells migrate in intestinal lymph under steady-state as well as under inflammatory conditions.

To further characterize the CD11c+ cells present in intestinal lymph, we established a novel technique, to isolate cells from murine intestinal lymph vessels for flow cytometric analysis. CX3CR1+/GFP mice received olive oil by gavage and, 1 h later, intestinal lymph vessels were carefully cannulated with a fine capillary. By collecting lymph from several vessels in each mouse, this method allowed us to isolate a total volume of ~0.2–0.4 µl of lymph per mouse (unpublished data). In the steady state, no CX3CR1high cells were detectable in intestinal lymph and <2% of all cells were CD11c+ MHCIIm cells or expressed intermediate levels of CX3CR1/GFP. Because of the low numbers of isolated cells, we were unable to further phenotype these populations. In contrast, 5 h after R848 administration up to 40% of all viable cells were CD11c+MHCIIm cells (Fig. 5 A). Importantly, such cells were not observed in intestinal lymph of chemokine receptor CCR7-deficient mice after R848 administration (Fig. 5 A), which is consistent with a central role of CCR7 for DC migration into draining LNs (Ohl et al., 2004; Johansson-Lindbom et al., 2005; Hintzen et al., 2006; Worbs et al., 2006). Pheno-typic analysis of CD11c+ cells demonstrated homogenous expression of CD103 and very weak expression of CX3CR1 and, hence, these cells phenotypically resembled intestinal CD103+ DC. In striking contrast to CD103+ DC, we failed to detect CX3CR1high cells in lymph preparations irrespective of R848 application. Furthermore, the frequency of CX3CR1int cells did not increase upon R848 treatment. Thus, in lymph isolated after R848 treatment <50 cells could be isolated expressing intermediate levels of CX3CR1, <20% (10 cells) of which coexpressed MHCIIm. Given the virtually complete absence of CX3CR1/GFP cells observed in lymph vessels by microscopy and the close association of CX3CR1+ cells with the lymph wall, it seems likely that these cells are contaminants from surrounding tissue. However we cannot, at this point, rule out that a few CX3CR1int cells expressing CX3CR1 levels too low to be detected by microscopy might enter intestinal lymph. Concomitant with the appearance of CD103+ DC in intestinal lymph, 16 h after oral R848 or i.p. LPS administration there was a marked decrease in CD103+ DC in the intestinal LP and accumulation of these cells in the MLN (Fig. 5 B and not depicted). In contrast, the numbers of CX3CR1high and CX3CR1int cells in the LP and MLN remained unchanged. In particular, no CX3CR1int cells could be observed in MLN irrespective of TLR stimulation. Finally, the inability of CX3CR1+ cells to migrate into lymph correlated with an inability of these cells to up-regulate CCR7 12 h after in vitro stimulation with LPS (Fig. S2).
Collectively, these results demonstrate that, in contrast to CD103+ DCs, CX3CR1+ LPCs are a nonmigratory population of cells in the LP and are thus unlikely to participate in the initiation of adaptive immune responses in intestinal draining LN.

**CX3CR1+ LPCs are inefficient at priming naive T cells in vitro and in vivo**

Besides their migratory properties, the most distinguishing feature of DCs is their potent antigen-presenting capacity. We therefore isolated CD103+ DC, CX3CR1+ LPC, and CX3CR1high LPC and analyzed their ability to present antigen to CD4+ and CD8+ T cells. Isolated cell populations were loaded in vitro with OVA peptide specific for MHC class I–restricted OT-I or MHCII-restricted OT-II T cells. As expected, peptide-loaded CD103+ DC induced efficient proliferation of OT-I and OT-II cells (Fig. 6 A; Johansson-Lindbom et al., 2005). In contrast, CX3CR1+ LPC showed a significantly reduced ability to activate OT-I and OT-II T cells and, in particular, OT-II. RAG−/− T cells. CX3CR1high LPC induced only very weak proliferation of OT-I and OT-II T cells and failed to induce OT-II. RAG−/− or OT-II proliferation (Fig. 6 A and not depicted). Notably, all three LPC populations expressed the costimulatory molecules CD40, CD80, and CD86 (Fig. S3 A). Under steady-state conditions, CD103+ DCs isolated from LP expressed lower levels of CD40 and CD86 compared with CD103+ DCs isolated from the MLN (unpublished data). This situation changed when CD103+ DCs were isolated from the LP after R848 treatment, resulting in increased expression of CD40 and CD86 (Fig. S3 B). However, such up-regulation was less evident on CX3CR1+ LPC (Fig. S3 B), and reduced induction of costimulatory molecules might at least partly explain the low ability of these cells to stimulate T cells.

Because in vitro peptide loading does not recapitulate the entire process of antigen uptake, processing, and presentation, we injected OVA-Cy5 into exteriorized intestinal loops of CX3CR1GFP/GFP mice to load cells with antigen in vivo. Flow cytometry revealed a higher proportion of antigen-positive CX3CR1+ LPC compared with CD103+ DC, indicating that CX3CR1+ LPC readily took up soluble fluorescently labeled OVA in vivo (Fig. 6 B). Nevertheless, in vivo–loaded CX3CR1+ LPCs were much less efficient in priming OT-II T cells compared with CD103+ DCs (Fig. 6 B). Notably, in CX3CR1GFP/GFP mice, lacking functional CX3CR1 protein, GFP+ LPC also took up fluorescently labeled OVA and adaptively transferred OT-II T cells were activated in the MLN of mice as efficiently as in WT recipients (Fig. S4, A and B). Because these mice fail to extend transmigrating dendrites (Niess et al., 2005), these results indicate that transmigrating dendrites are dispensable for uptake of and induction of T cell responses to oral soluble antigen.

Compared with the complex situation in an LN, in vitro co-cultures can only recapitulate some aspects of T cell activation. Therefore we investigated the antigen-presenting capacity of CD103+ DC compared with CX3CR1+ LPC in vitro and in vivo. (A) OT-I or OT-II cells were co-cultured with peptide-loaded CD103+ DC, CX3CR1+ LPC, or CX3CR1high LPC at a ratio of 10:1 for 3 d. Proliferation was determined by thymidine incorporation assay and is depicted as a percentage of the mean T cell proliferation in CD103+ DC co-cultures (thymidine index). Results are the mean (SEM) of three or more independent experiments using pooled cells from four to five mice per experiment. Data were analyzed using one sample Student’s t test, compared with CD103+ DC. Co-cultures of LPC/T cells at a 1:2 ratio showed a similarly inefficient induction of T cell proliferation compared with the 1:10 ratio (not depicted). *, P < 0.05; **, P < 0.01; ***, P < 0.001. (B) 500 µg of unlabeled or Cy5-labeled OVA was injected into the gut lumen of anaesthetized CX3CR1−/− mice. 1 h later, mice were sacrificed and Cy5 fluorescence in CD103+ DC and CX3CR1+ LPC was determined by flow cytometry. Dot plots are gated on CD103+ DC or MHCII+CX3CR1+ LPC as indicated. Gates were adjusted to contain 0.1% of positive cells in controls (injected with unlabeled OVA) and numbers indicate the proportion of Cy5-positive cells. Data are representative of five mice analyzed in three independent experiments. CD103+ DC and CX3CR1+ LPC from OVA-Cy5-injected mice were sorted 1 h after injection and co-cultured with OT-II T cells for 3 d at a ratio of 1:10. Cells were pulsed with 1 µCi [3H]thymidine 18 h before collection, and thymidine incorporation was quantified in a β counter. Bars represent pooled data obtained in two independent experiments. Error bars indicate SEM. Results were obtained with a one-sample Student’s t test. ***, P < 0.001. (C) 107 CFSE-labeled DO11.10 cells were adoptively transferred to BALB/c recipients. 1 d later, 5,000 peptide-loaded CD103+ DCs or CX3CR1+ LPCs were injected into the MLN–afferent lymphatics. Proliferating antigen–specific T cells were detected in the MLN 3 d after injection. Plots are gated on live CD4+ cells. Control mice received 20 mg OVA by gavage. Results are representative of two independent experiments.
vivo. Sorted CD103+ DC and CX3CR1+ LPC were loaded with OVA peptide specific for MHCII- restricted DO11.10 T cells, and 5,000 antigen-loaded cells were injected into the MLN-afferent lymph of recipient mice that previously received CFSE-labeled DO11.10 cells i.v. 3 d after intralymphatic injection, T cell proliferation was determined in MLN and peripheral LNs. Injection of CD103+ DC induced proliferation of antigen-specific DO11.10 T cells in the MLN but not in peripheral LNs (Fig. 6 C and not depicted). In contrast, antigen-loaded CX3CR1+ LPC failed to induce DO11.10 T cell proliferation in the MLN. Notably, the injected CX3CR1+ LPC included CX3CR1high and CX3CR1int LPC that showed weak but measurable CD4 T cell priming capacity in vitro (Fig. 6 A). Together, these results demonstrate that compared with CD103+ DCs, CX3CR1int LPCs and, in particular, CX3CR1high LPCs have only modest antigen-presenting capacity in vitro and in vivo.

**CX3CR1+ LPCs are not imprinted with an enhanced ability to metabolize vitamin A**

Given recent studies suggesting an important role for intestinal epithelial cells in imprinting CD11c+ with the ability to metabolize vitamin A and generate gut tropic T cells (Edele et al., 2008; Iliev et al., 2009), and the proximity of CX3CR1+ LPC to the intestinal epithelium, we assessed whether these cells were imprinted with the ability to generate RA. Real-time PCR analysis demonstrated that both CX3CR1int and CX3CR1high LPC expressed significantly lower levels of aldhl2 compared with CD103+ DC (Fig. 7 A). To assess whether such differences correlated with differences in aldehyde dehydrogenase (ALDH) activity, CD103+ DC and CX3CR1+ cells were incubated with the fluorescent ALDH substrate ALDEFLUOR, and ALDH activity was assessed by flow cytometry. Because the ALDEFLUOR signal emitted in the FITC channel, we focused on CX3CR1high cells that, in contrast to CX3CR1int, were readily identified in WT mice by gating on F4/80+MHCII+CD11b+high cells (Fig. S5). CD103+ DC exhibited significantly higher ALDH activity, compared with CX3CR1high cells, that was blocked with the ALDH inhibitor DEAB (diethylaminobenzaldehyde; Fig. 7, B and C). Finally, to assess whether these differences resulted in a reduced ability of CX3CR1+ LPC to induce CCR9 on responding T cells, CFSE-labeled OT-I cells were incubated together with peptide-pulsed CX3CR1high and CX3CR1int LPCs, and their expression of CCR9 was assessed by flow cytometry. In contrast to CD103+ DC (Fig. 7 D; Johansson-Lindbom et al., 2005; Jaenson et al., 2008), peptide-pulsed CX3CR1int and, in particular, CX3CR1high LPCs were inefficient at inducing CCR9 on responding T cells (Fig. 7 D). Together, these results suggest that CX3CR1+ LPCs are not locally imprinted with an enhanced ability to generate the vitamin A metabolite RA.

**DISCUSSION**

A paradigm has arisen during the last few years postulating that intestinal LPCs actively sample intestinal antigens through transepithelial dendrites and that such a process might drive the induction of intestinal immune responses in the gut draining MLN. Support for such a model came from numerous studies that, on the one hand, demonstrated transepithelial dendrite-forming cells in live tissue and on the other hand described functional properties of antigen-presenting cells in the LP and draining MLN. However, the interrelation of antigen-sampling cells in the LP and antigen-presenting cells in the MLN has not been directly investigated. Transepithelial dendrite-forming LPCs have mostly been visualized by GFP expressed under control of the CX3CR1 or CD11c promoter (Niess et al., 2005; Chieppa et al., 2006; Vallon-Eberhard et al., 2006), whereas CD103 has been used as a surrogate marker to identify LP-derived DC in the MLN (Annacker et al., 2005; Johansson-Lindbom et al., 2005).

In this study, we used immunofluorescence microscopy and flow cytometry to demonstrate that CD103+ DC and CX3CR1+ LPC represent phenotypically distinct cell populations in the LP. CX3CR1+ LPC outnumbered CD103+ cells three- to fourfold and, in contrast to CD103+ DC, localized close to the epithelium, which is consistent with previous results (Niess et al., 2005; Chieppa et al., 2006). CX3CR1+ LPC could be further divided into CX3CR1high and CX3CR1int LPC populations by flow cytometry. CX3CR1high and CX3CR1int LPCs exhibit differences in aldehyde dehydrogenase (ALDH) activity, as demonstrated by ALDEFLUOR assay. CX3CR1int LPCs have lower ALDH activity compared to CD103+ DC, suggesting a reduced ability to metabolize vitamin A. These differences are further supported by real-time PCR analysis, which shows lower aldhl2 expression in CX3CR1int LPCs compared to CD103+ DC. Additionally, peptide-pulsed CX3CR1int and CX3CR1high LPCs are inefficient at inducing CCR9 on responding T cells, as demonstrated by CFSE-labeled OT-I cells. This suggests that CX3CR1+ LPCs are not locally imprinted with an enhanced ability to generate the vitamin A metabolite RA.
LPCs, which represent the large majority of CX3CR1⁺ cells in the LP, appeared as a homogenous population of cells expressing CD11b, F4/80, and CD14, markers which are frequently used to identify macrophages. Although most CX3CR1int LPCs also expressed CD11b and F4/80, their variable expression of MHCII, CD11c, and CD14 suggested that this population consisted of several subsets of cells or cells at various stages of maturation.

In addition to their distinct phenotypes, CD103⁺ DC and CX3CR1⁺ LPC differed in their responsiveness to growth factors and turnover rates, suggesting that they derive from distinct precursors. CD103⁺ DC expanded in response to Flt3L and GM-CSF, whereas CX3CR1high LPC failed to expand in response to these cytokines. Moreover, CX3CR1⁺ cells showed slower turnover rates in vivo and, in contrast to CD103⁺ DC, were not replaced by recipient cells in small intestinal grafts. These results are consistent with two recent articles that were published while this manuscript was under review (Bogunovic et al., 2009; Varol et al., 2009). Bogunovic et al. (2009) demonstrated an important role for Flt3L and GM-CSF in the generation of CD103⁺ DC but not CD103⁻ MHCII⁺ CD11c⁺ CD11b⁻ cells, the large majority of which are CX3CR1⁺ (unpublished data), and showed that the former cells could derive from pre-DC, whereas the latter derive from Ly6C⁺ monocytes. Similarly, Varol et al. (2009) demonstrated that Flt3L expanded CD103⁺ CD11b⁻ DCs, the majority of which likely derive from intestinal isolated lymphoid tissues. However, their findings regarding responsiveness of CD11c⁺ CD11b⁻ cells to GM-CSF and Flt3L are difficult to interpret, as this population likely includes both CD103⁺ CD11b⁻ DCs, which make up the majority of CD103⁺ DCs in the LP, and CD11c⁻ CX3CR1⁺ LPCs. These authors also demonstrated that CX3CR1int and CX3CR1high cells derived from monocytes, whereas grafted pre-DCs predominantly gave rise to CD11c⁺ CX3CR1⁺ cells. It is of note that both these studies focused on the fraction of CX3CR1high cells that expressed sufficient levels of CD11c to fall within the CD11c⁺ MHCII⁺ high DC gate. However, our current results suggest that these cells are otherwise phenotypically identical to the CD11c⁻ CX3CR1high cells and should not be interpreted to represent classical DCs or a distinct subset of CX3CR1high cells.

Given previous studies suggesting that CX3CR1⁺ cells capture luminal antigen for initiation of immune responses (Niess et al., 2005; Chieppa et al., 2006; Niess and Reinecker, 2006; Vallon-Eberhard et al., 2006; Hapfelmeier et al., 2008; Uematsu et al., 2008) and indirect evidence indicating that at least a proportion of CD103⁺ MLN DC derive from the intestinal LP (Johansson-Lindbom et al., 2005; Hammerschmidt et al., 2008), we used two novel technical approaches, multi-photon confocal microscopy of intact lymph vessels and isolation of lymph cells, to determine whether these populations migrated in intestinal lymph. Using these methods, we provide direct evidence to suggest that CD103⁺ DCs are the dominant migratory DC population in the LP, whereas CX3CR1high LPCs represent a nonmigratory tissue resident population. Using confocal microscopy, no CX3CR1⁺ cells were observed in lymph under steady-state and inflammatory conditions. In contrast, CD11c⁺ cells were readily visible and dramatically increased in numbers after R848 administration. Flow cytometry confirmed the absence of CX3CR1high cells in intestinal lymph and revealed that the overwhelming majority of CD11c⁺ MHCII⁺ cells in lymph were CD103⁺ and, thus, indistinguishable from CD103⁺ DC in the LP. Consistently, oral administration of LPS (unpublished data) induced a loss of CD103⁺ DCs from the intestinal LP and a corresponding increase of these cells in the MLN, whereas CX3CR1⁺ cell numbers remained unchanged. These findings are consistent with studies in LysM-Cre x ROSA26stopflostopGFP mice, suggesting that the majority of CX3CR1⁺ cells in the MLN do not derive from the intestinal LP (Bogunovic et al., 2009), and previous studies examining lymph migrating DC in adrenalectomized rats and pigs (Pugh et al., 1983; Bimczok et al., 2005; Miller et al., 2006), indicating that only a subset of myeloid LPC is capable of entering draining lymph vessels (Bimczok et al., 2005; Yrlid et al., 2006). Moreover, they offer a plausible explanation for the failure in previous studies to observe epithelial-associated CD11c⁺ cells leaving the LP (Chieppa et al., 2006).

Although we failed to detect CX3CR1⁺ cells in lymph by confocal microscopy, CX3CR1int cells were detected in low but variable numbers in lymph preparations. We cannot at this point rule out that these few cells derive from the LP; however, CX3CR1⁺ cells were readily visualized in the fat tissue surrounding the lymph vessels and occasionally had protrusions extending into the lymph vessel. We thus assume that the few CX3CR1int cells in lymph preparations were contaminants from CX3CR1⁻ expressing cells in intimate contact with the vessel wall. Furthermore, the few MHCII⁺ cells within this population would seem highly unlikely to be in meaningful enough numbers to participate in initiation of T cell responses in the MLN.

To determine whether CX3CR1⁺ LPC and CD103⁺ DC displayed direct functional differences in their ability to drive characteristic intestinal T cell responses, we assessed their ability to prime T cell responses in vitro and in vivo. CD103⁺ DCs were more efficient antigen-presenting cells compared with CX3CR1int and CX3CR1high LPCs. Differences in antigen-presenting capacity were generally more pronounced in CD4⁺ compared with CD8⁺ T cell priming, in vivo versus in vitro assays, and CD103⁺ DC compared with CX3CR1high LPC. Moreover, these populations differed in their ability to generate RA, a central signaling molecule involved in shaping the signature of intestinal T cell responses. CX3CR1⁺ LPCs expressed significantly lower levels of aldha1a2, displayed low ALDH activity, and were poor at inducing CCR9 on responding T cells in comparison with CD103⁺ DC. These latter findings are somewhat surprising given the current dogma that DCs are conditioned by adjacent epithelial cells and recent studies suggesting that intestinal epithelial cells induce aldha1a2 on bone marrow-derived DCs and imprint them with an ability to induce
For the future, it will be important to interpret the function of myeloid LPCs based on the anatomical constraints of the intestinal immune system. CX3CR1+ LPCs capable of antigen presentation in vitro are unlikely to encounter naive T cell in vivo because naive T cells are mostly restricted to GALT and MLN and only pass through the LP very infrequently. In this regard, our use of CD103 and CX3CR1 as surrogate markers to define lymph migrating and gut-resident cells is likely to provide an important tool for further dissection of the functionality of LP subsets.

Although we suggest that CX3CR1+ LPCs do not act as classical DC-priming T cells in draining LN, our data do not rule out a potential role for these cells in modulating the adaptive immune responses. For example, CX3CR1+ LPCs that have been shown to engulf luminal bacteria (Niess et al., 2005; Hapfelmeier et al., 2008) and take up orally administered soluble proteins (this study) may directly pass antigenic material to CD103+ DC still localized in the LP. In this respect, a recent study reported localization of noninvasive S. enterica within CX3CR1+ cells in the cecum early after infection, whereas at later time points S. enterica were observed within CX3CR1+ cells (Hapfelmeier et al., 2008). Interestingly, uptake of soluble antigen from the intestinal lumen was intact in CX3CR1GFP/GFP mice, indicating that transepithelial extensions would not contribute to sampling of soluble antigen. Moreover, given recent results that antigen-presenting cells drive cytokine and proliferative responses of effector/memory T cells in peripheral tissues (Wakim et al., 2008; McLachlan et al., 2009), antigen presentation by CX3CR1+ LPC may regulate cytokine production and function of antigen-specific effector T cells subsequent to their entry into the intestinal LP. Alternatively, the central role of CX3CR1+ LPC may simply be to serve as a first line of defense by phagocytosing and killing bacteria on the epithelial surface or within the intestinal LP. Consistent with such a function, flagged S. enterica was recently shown to induce MyD88-dependent CX3CR1+ cell migration into the intestinal lumen, suggesting a role for these cells in cell-mediated immune exclusion (Arques et al., 2009).

In summary, our data suggest a division of labor between migrating LP DCs and gut-resident CX3CR1+ cells. CD103+ DCs are capable of intralymphatic migration and enter MLN. In the MLN, cell- and tissue-intrinsic influences, including RA, cooperate to shape the induction of intestinal immune responses. Although future studies are required to determine the potentially diverse in vivo functions of CX3CR1+ LPCs, the realization that these cells represent a gut-resident LP myeloid population will allow more focused efforts toward this goal.

MATERIALS AND METHODS

Mice. B6.129P-CX3CR1tm1mil/J (Jung et al., 2000), CX3CR11GFP mice on C57BL/6 background, BALB/c 129p. CX3CR1tm1mil/J (CX3CR11GFP/GFP and CX3CR11GFP/GFP mice on BALB/c background), B6.129P2(C)-Ccr7tm1Rfor/J (CCR7+/- mice [Forster et al., 1999]), C57BL/6-Tg(Tcra Tcrb)1100Mjbj (OT-I mice and OT-I mice crossed to RAG-2-deficient background), C57BL/6-Tg(Tcra Tcrb)425Cbn-Pprrc2 (OT-II Ly5.1 mice and OT-II

JEM VOL 206, December 21, 2009 3111
mice crossed to RAG-2-deficient background), and BALB/c-Tg(DO11.10) (DO11.10 mice), C57BL/6, BALB/c were bred at the central animal facility of Hannover Medical School and/or Biomedical Center animal facility (Lund University, Lund, Sweden) under specified pathogen-free conditions. CD11c-eYFP (Lindquist et al., 2004) mice were provided by T. Veres (Fraunhofer Institut für Toxikologie und Umweltforschung Medizin, Hannover, Germany). All animal experiments were performed in accordance with institutional guidelines and have been approved by the review board of Hannover Medical School and the Niedersächsische Landesamt für Verbraucherschutz und Lebensmittelsicherheit or by the Lund/Malmö Animal Ethics Committee.

Antibodies and reagents. The following antibodies and reagents were used: CCR9 (7E7; Pabst et al., 2004), CCR2 (MC-21; gift from M. Mack, Medical School and the Niedersächsische Landesamt für Verbraucherschutz und Lebensmittelsicherheit or by the Lund/Malmö Animal Ethics Committee.

Cell staining kit (Invitrogen). CFSE was obtained from Sigma-Aldrich. 7-amino-actinomycin d (Sigma-Aldrich), or Live Dead Fixable Violet Dead Nucleus VIII (Sigma-Aldrich) and 50 mM CaCl2 (W.W. Agace laboratory), the intestinal segment corresponding to distal jejunum and proximal ileum with 10% FCS, glutamine, and penicillin/streptomycin. Cells were washed thoroughly in PBS before transfection. 5 x 10^6–10^7 cells were injected subcutaneously into each dorsal flank of the mice. Cells were sacrificed after 6–12 d and analyzed by histology and/or flow cytometry.
Real-time PCR. Total RNA was isolated from LPC subsets using RNeasy Micro kit (QIAGEN) and complementary DNA was generated using Superscript III First-Strand kit (Invitrogen) with random hexamers. Expression of GAPDH and aldhl1a2 was analyzed using the MyQ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories), the Maxima SYBR Green qPCR kit (Fermentas), and the following primers: GAPDH forward, 5’-CCTGACACCAACACTGCTTA-3’; GAPDH reverse, 5’-TCATACCTGGCAGGTTCCTCCA-3’; aldhl1a2 forward, 5’-CAGATGCCATTTGACATCTAC-3’; and aldhl1a2 reverse, 5’-ATAAGTCCAGGATTGTTG-3’.

Statistical analysis. Statistical analysis was performed with Prism software (GraphPad Software, Inc.) using an unpaired two-tailed Student’s t test, a paired two-tailed Student’s t test, a one-sample Student’s t test, or a Wilcoxon signed rank test as indicated. Error bars represent SD or SEM as indicated. Statistical differences for the mean values are indicated as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Online supplemental material. Fig. S1 demonstrates the phenotype of CD103+ DC and CX3CR1+ LPC under steady-state conditions. Fig. S2 shows expression of CCR7 on CD103+ DC and CX3CR1+ LPC after LPS stimulation. Fig. S3 shows expression of costimulatory molecules on CD103+ DC and CX3CR1+ LPC under steady-state conditions and after R848 stimulation. Fig. S4 compares uptake of soluble OVA and activation of T cells in WT to CX3CR1 GFP/GFP mice. Fig. S5 illustrates gating of CX3CR1+ LPC independent of CX3CR1/GFP reporter activity. Video 1 and 2 illustrate occurrence of CX3CR1/GFP reporter activity.

We thank Drs. A. Mowat (Glasgow University), B. Johansson-Lindbom (Lund University), R. Förster, and G. Bernhard (both Hannover Medical School) for invaluable comments on the manuscript and support; Tiber Veres (ITEM, Hannover) for providing CD11c-YFP mice, and Anne-Charlotte Selberg for help with animal care.

This work was supported by grants to W.W. Agace from the Swedish Medical Research Council, the Crafoordiska, Österlund, Nanna Sverz and Kocks foundations, the Royal Physiographic Society, the Welcome Trust, UK and the Swedish foundation for Strategic Research FFL-II program, Deutsche Forschungsgemeinschaft SFB621-TP11 to O. Pabst, and Excellence Cluster REBIRTH. O. Schulz was supported by a scholarship from Hannover Biomedical Research School.

The authors have no conflicting financial interests.

Submitted: 3 September 2009
Accepted: 12 November 2009

REFERENCES


Figure S1. Characterization of CD103<sup>+</sup> DC, CX3CR1<sup>int</sup> LPC, and CX3CR1<sup>high</sup> LPC by flow cytometry. (A and B) CD103<sup>+</sup> DC, CX3CR1<sup>high</sup> LPC, and CX3CR1<sup>int</sup> LPC were analyzed for GFP reporter fluorescence (A) and expression of CD14, CD11b, F4/80, Ly6c, Gr-1, and CCR2 (B). Gray-filled histograms, staining; black line, isotype. In A, the black line indicates autofluorescence detected in the FITC/GFP channel in CD103<sup>+</sup>MHCII<sup>+</sup>CD11c<sup>+</sup> LPCs isolated from WT mice. Representative FACS histograms/plots are shown from three or more independent experiments. (C) CX3CR1<sup>int</sup> LPC contained a subpopulation of MHCII<sup>−</sup>Gr-1<sup>+</sup> cells that also expresses CD11b, CD62L, and CCR2 but was negative for CD11c. Representative FACS histograms/plots are shown from two to seven mice analyzed in two to three independent experiments.
Figure S2. CD103⁺ DCs, but not CX3CR1⁺ LPCs, express CCR7 after in vitro stimulation. Cells were isolated from the LP of CX3CR1⁺GFP mice and incubated for 12 h in the presence of 1 µg/ml LPS. CCR7 was measured by flow cytometry, and CCR7 fluorescence is depicted as gray-filled histograms and isotype control staining as solid lines. Data shown are representative of two independent experiments.

Figure S3. Expression of costimulatory molecules on LPC in the steady-state and after R848 administration. LP and MLN cells were isolated from naïve mice (A) or 16 h after oral administration of R848 (B), and expression of costimulatory molecules on LPC subsets and CD103⁺ MLN DC was assessed by flow cytometry. Gray-filled histograms show specific antibody staining and unfilled histograms show the signal of appropriate isotype controls. Four mice per group were analyzed in two independent experiments.
Figure S4. Uptake of soluble OVA and activation of T cells is normal in CX3CR1\(^{GFP/GFP}\) mice. (A) 500 µg of Cy5-labeled OVA or unlabeled OVA was injected into the gut lumen of anesthetized CX3CR1\(^{GFP/GFP}\) mice. 1 h later, mice were sacrificed and Cy5 fluorescence in CX3CR1\(^{+}\) LPC was determined by flow cytometry. Dot plots are gated on MHCII\(^{-}\)CX3CR1\(^{+}\) LPC, gates were adjusted to contain 0.1% of positive cells in controls (injected with unlabeled OVA), and numbers indicate the proportion of Cy5-positive cells. Results are representative stainings from one experiment of two performed. (B) CFSE-labeled DO11.10 cells were adoptively transferred into WT BALB/c and CX3CR1\(^{GFP/GFP}\) mice lacking functional CX3CR1 receptor and, thereby, transepithelial dendrites. Mice received 50 mg OVA orally 4 h after transfer. 3 d after OVA application, cells were isolated from the MLN and analyzed for T cell activation by flow cytometry. Results are representative of three experiments performed with at least two mice each. Error bars represent SD.
Figure S5. Gating strategy for CX3CR1\textsuperscript{high} LPC in WT mice. ALDEFLOUR measurements were performed on LPC preparations from WT mice using an alternative gating strategy to identify CX3CR1\textsuperscript{high} LPC. Comparison of LPC staining of WT (top) and CX3CR1\textsuperscript{+/GFP} mice (bottom) demonstrates that >95% of F4/80\textsuperscript{+}MHCII\textsuperscript{+}CD11b\textsuperscript{+} cells were CX3CR1\textsuperscript{high} in CX3CR1\textsuperscript{+/GFP} mice. Results are representative of two experiments performed with at least two mice each.

Video 1. Two-photon microscopy of intestinal lymphatics of CX3CR1\textsuperscript{+/GFP} mice in the steady state. The vessel lumen was visualized with TRITC-labeled dextran injected i.v. 30 min before analysis (red) and the second harmonic signal of the vessel wall (blue). Grid size, 50 µm. The projection shown in Fig. 4B (top right) was generated from this volume. CX3CR1/GFP\textsuperscript{+} cells (green) can be observed surrounding intestinal lymph vessels but not in the vessel lumen.

Video 2. Two-photon microscopy of intestinal lymphatics 5 h after oral administration of 10 µg R848 to CD11c-EYFP mice. TRITC-labeled dextran (red) was injected i.v. 30 min before analysis. The second harmonic signal of the vessel wall is displayed in blue and CD11c/YFP\textsuperscript{+} cells in green/yellow. Grid size, 50 µm. The projection shown in Fig. 4B (bottom left) was generated from this volume. Numerous CD11c/YFP\textsuperscript{+} cells are readily observed in the vessel lumen.