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CCR7 Is Critically Important for Migration of Dendritic Cells in Intestinal Lamina Propria to Mesenteric Lymph Nodes¹

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Although dendritic cells (DCs) located in the small intestinal lamina propria (LP-DCs) migrate to mesenteric lymph nodes (MLNs) constitutively, it is unclear which chemokines regulate their trafficking to MLNs. In this study we report that LP-DCs in unperurbed mice require CCR7 to migrate to MLNs. In vitro, LP-DCs expressing CCR7 migrated toward CCL21, although the LP-DCs appeared morphologically and phenotypically immature. In MLNs, DCs bearing the unique LP-DC phenotype (CD11c^{high}CD8 α ^{int}CD11b^{low} α _L^{low} β ₇^{high} and CD11c^{high}CD8 α ⁻CD11b^{high} α _L^{low} β ₇^{high}) were abundant in wild-type mice, but were markedly fewer in CCL19-, CCL21-Ser-deficient *plt/plt* mice and were almost absent in CCR7-deficient mice, indicating the critical importance of CCR7 in LP-DC trafficking to MLNs. Interestingly, CCR7⁺ DCs in MLNs with the unique LP-DC phenotype had numerous vacuoles containing cellular debris in the cytoplasm, although MLN-DCs themselves were poorly phagocytic, suggesting that the debris was derived from the LP, where the LP-DCs ingested apoptotic intestinal epithelial cells (IECs). Consistent with this, LP-DCs ingested IECs vigorously in vitro. By presenting IEC-associated Ag, the LP-DCs also induce T cells to produce IL-4 and IL-10. Collectively, these results strongly suggest that LP-DCs with unique immunomodulatory activities migrate to MLNs in a CCR7-dependent manner to engage in the presentation of IEC-associated Ags acquired in the LP. *The Journal of Immunology*, 2006, 176: 803–810.

Dendritic cells (DCs)⁴ are cardinal constituents of the immune system and play pivotal roles in the induction of Ag-specific immune responses and the maintenance of self-tolerance (1). DCs are abundant in the small intestine, both in organized lymphoid tissues (Peyer's patches (PPs) and isolated lymphoid follicles (ILFs)) and in the lamina propria (LP), the layer of connective tissue between the epithelium and the muscularis mucosa, where they act as sentinels for incoming Ags. The precise discrimination between harmless Ags and dangerous pathogens by these DCs is a likely key mechanism for the maintenance of gut immune homeostasis (2).

Among the intestinal DCs, the DC subsets in PPs have been characterized in the most detail (3–6). PP-DCs can educate Ag-specific T cells to produce IL-4 and IL-10 (3) and confer gut-homing specificity on T cells (7, 8), indicating that they have unique immune-inductive abilities. In contrast, LP-DCs have been only incompletely characterized, mainly due to difficulty in isolating them. Recent investigations have revealed, however, that LP-DCs of a certain subset extend dendrites in a CX₃CR1-dependent manner to the luminal side of the gut for the uptake of Ags (9, 10). In addition, LP-DCs that reside in the terminal ileum sample commensal bacteria and constitutively express IL-12 p40, indicating that these LP-DCs may be involved in the predisposition to chronic inflammation (11). LP-DCs may also be important in the presentation of bacterial Ags directly to LP B cells (12, 13). LP-DCs obtained from mice treated with Flt3 (FMS-like tyrosine kinase 3) ligand, express high levels of IL-10 and type I IFN and can induce a state of immune hyporesponsiveness upon in vivo transfer (14), suggesting that LP-DCs may have an immunomodulatory role in the gut.

Apart from the LP-DCs, a distinct DC subset has been documented in rat intestinal lymph that can constitutively endocytose apoptotic intestinal epithelial cells (IECs) and transport them to the T cell areas of mesenteric lymph nodes (MLNs). However, it remains unclear whether these DCs are derived from the LP, PPs, and/or other intestinal compartment(s). In addition, their function remains unexplored, although they have been implicated in tolerance induction (15). It has been separately reported that among DCs in the MLNs, the CD8 α ⁻CD11b⁺ DC subset plays a critical role in inducing cross-tolerance to food Ags, although it remains to be determined whether these DCs take up dying IECs (16).

DCs are thought to leave peripheral tissues when they receive an inflammatory or danger signal. During this process, DCs begin to mature, and the expression of CCR7 increases (17–19), which allows the DCs to enter lymph vessels and gain access to T cell areas

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⁴ Abbreviations used in this paper: DC, dendritic cell; IEC, intestinal epithelial cell; ILF, isolated lymphoid follicle; LN, lymph node; LP, lamina propria; MLN, mesenteric lymph node; PP, Peyer's patch; SP-DC, splenic DC.

in draining lymph nodes (LNs) in a CCR7-dependent manner (20). Corroborating this, a deficiency of CCR7 or its ligands, CCL19 and CCL21, leads to impaired DC migration into draining LNs and abnormal lymph node architecture in peripheral tissues (21, 22). In addition, a recent study indicates that steady-state trafficking of skin DCs to the draining LNs in peripheral tissues is also regulated by CCR7-mediated signaling (23). However, it is not known whether this commonly held paradigm of DC trafficking being driven by CCR7-mediated signaling holds true for DCs in the intestinal compartment as well.

In the present study we found that there are at least two LP-DC subsets in the intestinal LP of unperturbed mice and that they both require CCR7 for their constitutive migration to the MLNs. They vigorously ingest apoptotic IECs and, hence, are likely to correspond to the cells identified by Huang et al. (15) in rat mesenteric lymph. As suggested previously (15), LP-DCs can present IEC-associated Ags to CD4⁺ T cells, inducing their differentiation into IL-4- and IL-10-producing cells. These results strongly indicate that LP-DCs bearing a unique immunomodulatory activity migrate constitutively to MLNs in a CCR7-dependent manner, thus generating a noninflammatory environment in the MLNs.

Materials and Methods

Mice

BALB/c mice were obtained from CLEA Japan. C57BL/6 mice were obtained from Japan SLC. Mice transgenic for a TCR that recognizes the OVA_{323–339} peptide in the context of I-A^d (DO 11.10 TCR- $\alpha\beta$ transgenic mice) on the BALB/c background were a gift from Dr. S. Ono (Osaka University Graduate School of Medicine, Osaka, Japan). The *pltp/ptp* mice on the B6 background were provided by Dr. H. Nakano (Duke University Medical Center, Raleigh, NC). CCR7-deficient mice on the C57BL/6 background were produced as previously described (21). All animal experiments were performed under an experimental protocol approved by the ethics review committee for animal experimentation of Osaka University Graduate School of Medicine.

Preparation of DCs from small intestinal LP, PPs, MLNs, and spleen

Small intestinal segments and PPs were treated with PBS containing 10% FCS, 20 mM HEPES, 100 U/ml penicillin, 100 μ g/ml streptomycin, 1 mM sodium pyruvate, 10 mM EDTA, and 10 μ g/ml polymyxin B (Calbiochem) for 30 min at 37°C to remove epithelial cells and were washed extensively with PBS. Small intestinal segments, PPs, MLNs, and spleen were digested with 400 Mandl units/ml collagenase D (Roche) and 10 μ g/ml DNase I (Roche) in RPMI 1640/10% FCS with continuous stirring at 37°C for 45–90 min. EDTA was added (10 mM final concentration), and the cell suspension was incubated for an additional 5 min at 37°C. Cells were spun through a 15.5% Accudenz (Accurate Chemical & Scientific) solution to enrich for DCs. The obtained cells were incubated with FITC-conjugated anti-CD11b and PE-conjugated anti-CD11c after FcR blocking. DC subsets were sorted on the basis of their expression of CD11c and CD11b by FACS Vantage SE (BD Biosciences). The purity of the sorted DCs was routinely >95%. For the morphological study, cytospin preparations from purified DC subsets were stained with May-Grunwald-Giemsa solution.

Double immunofluorescence staining of small intestinal LP

To determine the location of LP-DCs in the small intestinal LP, biotinylated anti-CD11c and FITC-conjugated anti-CD11b mAbs were applied overnight at 4°C to sections cut from frozen tissue. Samples were washed and then incubated with streptavidin-Alexa 594 (Molecular Probes) for 2 h at room temperature. To detect CCR7⁺ DCs in the small intestinal LP, frozen sections of the small intestine were stained with rabbit anti-mouse CCR7 pAb, provided by Dr. K. Matsushima (University of Tokyo School of Medicine, Tokyo, Japan) and biotinylated anti-CD11c mAb overnight at 4°C. The sections were washed and then further incubated with Alexa 488-conjugated chicken anti-rabbit IgG Ab (Molecular Probes) and streptavidin-Alexa 594 for 2 h at room temperature. Immunohistochemical staining was analyzed with a Radiance 2100/Bio-Rad confocal laser microscope (Bio-Rad).

Flow cytometry

Fluorochrome-conjugated anti-CD11c (HL3), anti-CD11b (M1/70), and anti-CD8 α (53-6.7) mAbs were used for DC staining. Anti-mouse CD16/CD32 (2.4G2) was used for FcR blocking. The expression of integrins was determined using mAbs to integrin α_L (CD11a; M17/4) and β_7 (M293). The expression of costimulatory molecules was determined using mAbs to B7-1 (CD80; 16-10A1), B7-2 (CD86; GL1), CD40 (3/23), and I-A^d (AMS-32.1). These reagents were all purchased from BD Pharmingen. After the FcRs were blocked for 15 min at 4°C, the cells were stained for integrins, CD8 α , CD11c, CD11b, and costimulatory molecules and then analyzed with a FACSCalibur (BD Biosciences). The DCs were identified by gating on the CD11c^{high} cells. CCR7 expression by DCs was determined using the CCL19-Fc chimeric protein provided by Drs. K. Hieshima and O. Yoshie (Kinki University School of Medicine, Kinki, Japan).

RT-PCR for chemokine receptor expression

Total RNA was prepared from freshly isolated LP-DC using TRIzol (Invitrogen Life Technologies). RT of total RNA was conducted using oligo(dT)₁₈ primer and SensiScript reverse transcriptase (Qiagen). PCR was conducted using primer pairs for CCR1 (sense, AGAAGCCTACCCCA CAAC; antisense, TGGCCAGGTATCTGTCAA), CCR7 (sense, GGTGT GCCTCTGCCAAGA; antisense, TGCCAAAGATGCCCTTAC), CCR9 (sense, TGCTACTGGAGACAACCTCG; antisense, CTCCTCAGAAGT GCAGTTAC), and β -actin (sense, ATGGATGACGATATCGCT; antisense, ATGAGGTAGTCTGTCAGGT) and Ex-Taq polymerase (Takara Shuzo). The PCR conditions were 30 cycles at 97, 57, and 72°C for 30 s each, and the products were analyzed on agarose gels.

Chemotaxis assays

All cell suspensions and chemokine dilutions were made in RPMI 1640 containing 0.5% low endotoxin BSA (Sigma-Aldrich). The chemokine CCL21 was purchased from Techne. Chemotactic assays were performed as previously described (24). Two hours after the start of migration, the inserts were removed. Migrated DCs were identified on a FACSCalibur using FITC-conjugated anti-CD11b mAb and PE-conjugated anti-CD11c mAb.

Real-time chemotaxis assay

Real-time chemotaxis assays were performed as previously described (25). To count the migrated cells in each channel, images of the cells in each channel were digitally recorded onto a computer hard disk with time-lapse intervals of 60 s.

Detection of apoptotic IECs in MLN-DCs

To detect IECs-derived apoptotic DNA in DCs, cytospin preparations from FACS-sorted MLN-DCs were fixed with 1% paraformaldehyde and stained using an ApopTag peroxidase in situ apoptosis detection kit (Serologicals). Detection of alkaline phosphatase activity in the FACS-sorted CD11c^{high}CD8 α ^{int} α_L ^{low} β_7 ^{high} MLN-DC subset was performed using the Vector Red Alkaline Phosphatase Substrate Kit I (Vector Laboratories).

Electron microscopy

Isolated cells were spun at 400 \times g and fixed in 1% in glutaraldehyde in 0.1 M phosphate buffer for 1 h at 4°C. After being washed, the cells were embedded in 2% agarose gel and postfixed in 2% osmium tetroxide in 0.1 M phosphate buffer for 2 h at 4°C. The fixed samples were then dehydrated in a graded ethanol series, infiltrated with propylene oxide, and embedded in Quetol 812 epoxy resin. Ultrathin sections were stained with 2% uranyl acetate and Reynold's lead citrate, then examined using a JEOL JEM-1230 electron microscope.

In vitro uptake of CFSE-labeled apoptotic epithelial cells by DCs

Small IECs were obtained as described previously (26). IECs were then labeled with CFSE (Molecular Probes) at a concentration of 5 μ M for 5 min at 37°C and cultured for 4 h to induce spontaneous apoptosis. Enriched DCs were mixed with CFSE-labeled apoptotic IECs and cultured for 4 h at 37°C. After the coculture, cells were stained with PE-conjugated anti-CD11c mAb and allophycocyanin-conjugated anti-CD11b mAb to identify the DC subsets. The uptake of apoptotic IECs by DCs was evaluated by FACSCalibur.

Analysis of CD4⁺ T cell proliferation and cytokine production

IECs obtained as described above were loaded with OVA (Sigma-Aldrich) by osmotic shock (27). They were then cocultured with DCs for 4 h, and

the DC subsets were subsequently sorted on the basis of their expression of CD11c and CD11b. Purified DCs (5×10^3) and DO11.10 OVA TCR-transgenic T cells (1×10^5) were mixed in 96-well plates (1/20). After 7 days, the cells were collected and stained with anti-mouse DO11.10 TCR (KJ1-26; Caltag Laboratories) and anti-mouse CD4 (RM4-5) mAbs. Dead cells were excluded using 7-aminoactinomycin D (Sigma-Aldrich). T cell proliferation was measured by CFSE dilution. To examine cytokine secretion, DO11.10 CD4⁺ T cells were cocultured with IEC-OVA-containing DCs for 14 days (two-round stimulation with IEC-OVA-containing DCs with a 7-day interval). The T cells were then washed and restimulated for 6 h with anti-CD3 mAb in the presence of monensin (BD Pharmingen). Intracellular cytokine staining was performed according to the manufacturer's instructions (BD Pharmingen).

Results

Small intestinal LP contains two distinct DC subsets

We first determined the localization of LP-DC subsets in the small intestine by immunohistochemistry. As shown in Fig. 1A, cells that were CD11b⁻/CD11c⁺ (open arrowheads) and CD11b⁺/CD11c⁺ (filled arrowheads) were readily recognizable in the LP, indicating that the small intestinal LP contains at least two phenotypically distinguishable DC subsets.

We next attempted to isolate LP-DCs and successfully obtained substantial numbers of low density leukocytes from the LP ($1.95 \pm 0.5 \times 10^6$ cells/mouse; $n = 34$), of which the CD11c⁺ DCs constituted ~10–15%. Among these cells, at least two LP-DC subsets could be recognized on the basis of their different CD11c/CD11b expression patterns: CD11c^{high}CD11b^{low} (R1; $4.9 \pm 2.0\%$ of low density cells) and CD11c^{high}CD11b^{high} (R2; $8.4 \pm 2.0\%$; Fig. 1B). The remaining cells, which were CD11c^{int}CD11b^{high}, consisted mainly of cells that contained eosinophilic granules (J.-H. Seoh and M. H. Jang, manuscript in preparation). The CD11c^{high} LP-DC subsets were heterogeneous in their CD8 α expression, in that the CD11c^{high}CD11b^{low} subset expressed CD8 α at an intermediate level (CD8 α ^{int}), whereas the CD11c^{high}CD11b^{high} subset was CD8 α ⁻ (Fig. 1B). Thus, the phenotypes of the R1 and R2 subsets were CD11c^{high}CD8 α ^{int}CD11b^{low} and CD11c^{high}CD8 α ⁻CD11b^{high}, respectively.

These two subsets were found not only in the intestines of Id2^{-/-} mice, which are completely deficient in PPs and ILFs (28), but also in the BALB/c small intestine from which PPs and ILFs had been surgically removed before the isolation procedure (data not shown), suggesting that they are indeed derived from the small intestinal LP and not PPs or ILFs. Interestingly, the freshly isolated R1 and R2 subsets both had few dendrites. The nuclear chromatin was not very condensed, and the cytoplasm was light blue to grayish when stained with May-Grunwald-Giemsa solution, suggesting that the LP-DCs were not fully mature (Fig. 1C).

The expression of costimulatory molecules also supported the idea that these cells were somewhat immature. As shown in Fig. 1D, LP-DCs showed substantially lower expression of MHC class II and CD40 than DCs from PPs and MLNs, indicating that they were less mature than other DCs in the intestinal compartment. Interestingly, however, the LP-DCs displayed a relatively high expression level of B7-2, as seen in PP-DCs, if not as high as that expressed by MLN-DCs, indicating that the LP-DCs may not be entirely immature but, rather, may constitute semimature subsets. The CD11c^{high} LP-DC subsets were DEC-205⁺, but B220⁻ and Gr-1⁻ (data not shown), indicating that they are distinct from plasmacytoid DCs (29) or the recently identified CD70⁺ APCs (30) in the LP, both of which express readily detectable levels of DEC-205, B220, and Gr-1.

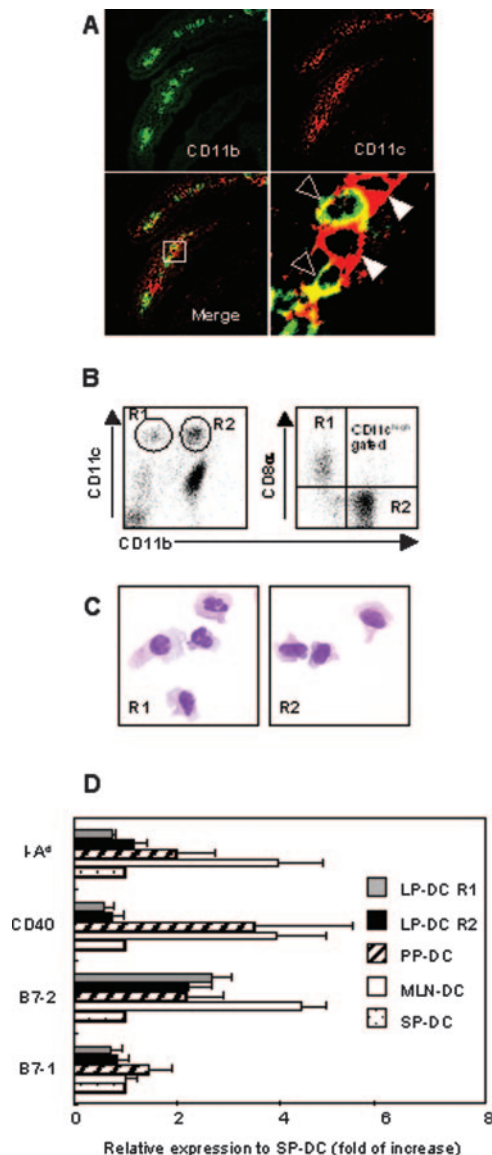


FIGURE 1. Identification of two DC subpopulations in the LP of the small intestine. *A*, Frozen sections of small intestine were fixed, stained with Abs specific for CD11b (green) and CD11c (red), and analyzed by confocal microscopy. Two cell subsets, CD11b⁻CD11c⁺ (red; open arrowheads) and CD11b⁺CD11c⁺ (yellow; filled arrowheads) were readily identifiable within the LP. *B*, Low density lamina propria cells were isolated from the small intestines of BALB/c mice and spun through a 15.5% Accudenz gradient. Enriched DCs were stained for CD8 α , CD11b, and CD11c and analyzed by flow cytometry. *C*, Two DC subsets (R1 (CD11c^{high}CD8 α ^{int}CD11b^{low}) and R2 (CD11c^{high}CD8 α ⁻CD11b^{high})) were FACS-sorted based on their CD11c and CD11b expressions and stained with May-Grunwald-Giemsa. The R1 and R2 subsets had a morphology associated with highly motile cells. *D*, The LP-DC subsets had a semimature phenotype. DC subsets from LP, PPs, MLNs, and spleen (SP) were stained for CD11c, CD11b, and B7-1, B7-2, CD40, or I-A^d and analyzed by flow cytometry. The expression levels are shown as the ratio of the mean fluorescence intensity (MFI) to the fluorescence intensity of SP-DCs.

LP-DC subsets express CCR7 and show directional migration toward CCL21

Although steady-state trafficking of DCs from the skin to the draining LNs is regulated by CCR7-mediated signaling (23), it remains to be established whether LP-DC trafficking is also regulated by a

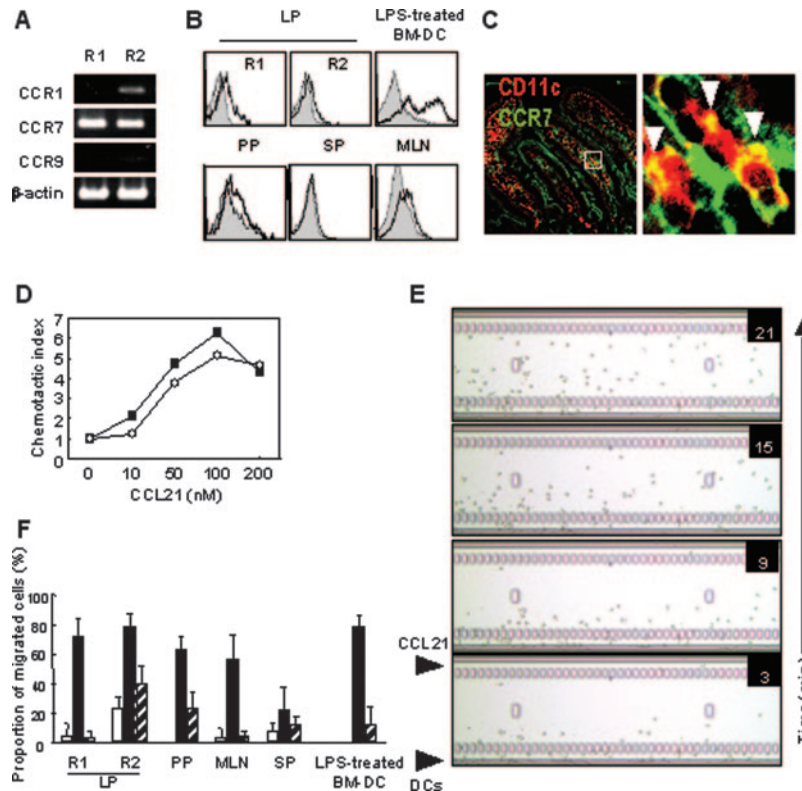


FIGURE 2. LP-DC subsets express CCR7 and show directional migration toward CCL21. *A*, Expression of CCR7 mRNA. cDNA was prepared from total RNA obtained from freshly FACS-sorted LP-DCs, and the expression of chemokine receptors was analyzed by semiquantitative PCR. *B*, Expression of CCR7 protein on the cell surface. DCs were stained for CCR7 using the CCL19-Fc chimera protein (□). LPS-stimulated bone marrow-derived DC was used as a positive control, and human Ig Fc protein served as a negative control (■). *C*, Localization of CCR7⁺/CD11c⁺ DCs in the small intestinal LP. Frozen sections of the small intestine were fixed and stained with anti-CCR7 pAb and biotinylated anti-CD11c mAb. The sections were further incubated with Alexa 488-conjugated secondary Ab and streptavidin-Alexa 594. Arrows indicate CCR7⁺/CD11c⁺ cells. *D*, Chemotaxis analysis by Transwell. LP-DCs were placed in the upper well of a Transwell apparatus (5- μ m pore size), and an increasing concentration of CCL21 was added to the lower well for 2 h. The migrated cells were stained for CD11c and CD11b, then analyzed by FACSCalibur. The proportion of migrated cells in each population was calculated as a fraction of the input population. The chemotactic index is shown as the ratio of the proportion of cells that migrated in the presence of chemokine to the proportion that migrated in the absence of chemokine. ■, R1 subset; ○, R2 subset. *E*, Time-lapse video monitoring of chemotaxis. Isolated LP-DCs were applied to the microchemotaxis chamber. After aligning the cells on the edge of the microchannel of the chamber, CCL21 (10 μ M) was applied to the opposite side of the microchannel (see *top* of each frame), so that a concentration gradient of the chemokine formed from the top to the bottom of the channel. The migration of cells in the microchannel was subsequently monitored at 6-min intervals. Note that a significant fraction of the cells had begun migrating from the bottom to the top of the field 15–21 min after the addition of CCL21. *F*, Quantitative evaluation of chemotactic responses to CCL21. Data are shown as the proportion of cells that migrated across the microchannel to the total cells in the assay area. □, Medium alone; ■, CCL21; ▨, CCL21 with PTX treatment. The data are representative of at least three independent experiments.

CCR7-dependent mechanism. We thus examined CCR7 expression in LP-DCs. In the R1 (CD11c^{high}CD11b^{low}) and R2 (CD11c^{high}CD11b^{high}) subsets, CCR7 mRNA was highly expressed, whereas CCR9 mRNA was absent (Fig. 2*A*). Consistent with this, LP-DCs were CCR7⁺, as evidenced by their binding of a CCL19-fusion protein (Fig. 2*B*), and CD11c⁺ DCs expressing CCR7 were readily detectable in the LP by immunohistochemistry (Fig. 2*C*). Furthermore, *in vitro*, the CD11c^{high} LP-DCs efficiently migrated through Transwell inserts in response to CCL21 with the typical bell-shaped dose-response curve that is characteristic of chemotaxis (Fig. 2*D*). To verify that this reflects directional, but not random, migration, we adopted an optical chemotaxis assay system that allows time-lapse video monitoring of cell behavior in silicon-coated microchannels (25). As shown in Fig. 2*E* and supplemental movie A,⁵ LP-DCs moved swiftly along the CCL21 concentration gradient, verifying that they can migrate directionally toward a CCL21 source as a result of expressing functional CCR7. Compared with DCs from other tissues, LP-DCs showed

much stronger chemotaxis toward CCL21 than splenic DCs (SP-DCs), but their chemotaxis was comparable to that seen in MLN-DCs and PP-DCs (Fig. 2*F*). LPS-stimulated, bone marrow-derived DCs responded to CCL21 much like the LP-DC subsets. In all these cell types, CCL21-mediated migration was significantly blocked by pertussis toxin. These data indicate that LP-DCs with relatively immature morphology and phenotype can migrate toward CCL21 as efficiently as mature DCs without deliberate inflammatory stimulation.

LP-DCs migrate to MLNs in a CCR7-dependent manner

We next investigated whether LP-DCs could be identified in the MLNs and, if so, whether their migration was dependent on CCR7. Because a recent study indicated that DC migrants to lymph nodes could be discriminated by their surface phenotype (31), we first compared the expressions of various surface markers on the CD11c^{high} DCs obtained from the LP, PPs, MLNs, and spleen. As shown in Fig. 3*A*, LP-DCs highly expressed β_7 integrin compared with other DCs. In this regard, LP-DCs contained CD8 α ^{int} β_7 ^{high} and CD8 α ⁻ β_7 ^{high} subpopulations, which correspond to the R1 and

⁵ The online version of this article contains supplemental material.

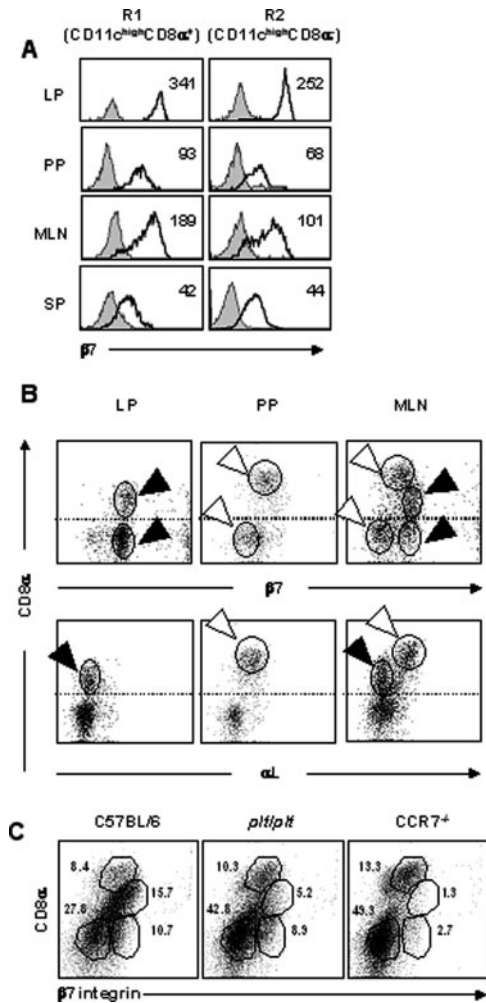


FIGURE 3. Apparent CCR7 dependency of the CD8α^{int}β₇^{high} and CD8α⁻β₇^{high} subsets in MLNs. *A*, LP-DCs highly expressed β₇ integrin compared with other DCs. Low density cells from the LP were stained for CD11c, CD8α, and β₇ integrin, and the histogram profiles were acquired after gating on CD11c^{high}CD8α⁺ DCs (R1) or CD11c^{high}CD8α⁻ DCs (R2). □, Isotype controls; ▣, stained cells. The numbers in the histograms indicate the MFI. *B*, LP-DCs and PP-DCs appear to constitute the MLN-DCs. Low density cells from the LP were double stained for CD8α/integrin β₇ or for CD8α/integrin α_L. FACS profiles were acquired after gating on the CD11c^{high} cells. Based on the CD8α/integrin β₇ double staining, the MLN-DCs consisted of four recognizable subsets, with two of them phenotypically corresponding to LP-DCs (filled arrowheads) and the remaining two corresponding to PP-DCs (open arrowheads). A similar observation was made with CD8α/integrin α_L double staining; MLN-DCs showed two CD8α⁺ subsets, one of which corresponded phenotypically to an LP-DC subset (filled arrowhead) and the other to a PP-DC subset (open arrowhead). *C*, CD8α^{int}β₇^{high} and CD8α⁻β₇^{high} LP-DCs were significantly fewer in the MLNs of *plt/plt* mice and were almost absent from the MLNs of CCR7-deficient mice. Numbers indicate the percentage of each subset within gated CD11c^{high} cells.

R2 subsets, respectively, and the PP-DCs had CD8α^{high}β₇^{int} and CD8α⁻β₇^{low} populations. In contrast, the MLN-DCs contained all four surface phenotypes, supporting the possibility that LP-DCs and PP-DCs enter the MLNs to make up the four different DC subsets there (Fig. 3*B*). Furthermore, the examination of α_L expression in CD8α⁺ DCs revealed that those in the LP expressed relatively low levels of α_L (CD8α^{int}α_L^{low}), whereas those in PPs expressed high levels of α_L (CD8α^{high}α_L^{high}), and the MLN-DCs consisted of both populations (Fig. 3*B*). These observations thus

suggest that the MLNs collect phenotypically different DCs from the LP and PPs, in accordance with the MLNs being the draining LNs of the LP and PPs.

We next asked whether these intestinal DCs enter the draining MLNs in a manner dependent on CCR7. For this purpose, we examined MLN-DCs in wild-type C57BL/6 mice; in C57BL/6-*plt/plt* mice, which are deficient in CCL19/CCL21-Ser (22); and in CCR7-deficient mice (21) that had been backcrossed to the C57BL/6 genetic background. As shown in Fig. 3*C*, the CD8α^{int}β₇^{high} and CD8α⁻β₇^{high} subsets (R1 and R2 in the LP, respectively) represented subsets of the MLN-DCs of wild-type mice (15.7 and 10.7% of the total CD11c^{high} DCs, respectively), but these subsets were significantly less prominent (5.2 and 8.9%) in the MLNs of *plt/plt* mice, which lack CCL21-Ser, but express CCL21-Leu, in their lymphatics, and were almost totally absent (1.3 and 2.7%) in the MLNs of CCR7-deficient mice. These results strongly suggest that both the R1 and R2 LP-DC subsets migrate to the MLNs in a CCR7-dependent manner under steady-state conditions. Consistent with this, both these LP-DC subsets were present in the intestine of CCR7-deficient mice (M. H. Jang and N. Sougawa, unpublished observation).

We also found that the majority of MLN-DCs were CCR7⁺, as evidenced by their binding of a CCL19 fusion protein (Fig. 2*B*), and like the unique DCs documented in rat intestinal lymph by Huang et al. (15), MLN-DCs contained much debris in the cytoplasm (Fig. 4*A*). The pieces of debris were TUNEL positive, that is, they showed apoptosis-induced DNA fragmentation (Fig. 4*B*). In addition, a considerable proportion of these cells contained granules that were positive for alkaline phosphatase (Fig. 4*C*), which is expressed in epithelial cells, but not DCs. In contrast, the MLN-DCs themselves showed little phagocytic activity, as described below. These results are compatible with the idea that the CCR7⁺ MLN-DCs were derived from the LP in a CCR7-dependent manner, after having ingested apoptotic IECs in the LP.

LP-DCs efficiently endocytose apoptotic epithelial cells

Because IECs undergo apoptosis extensively in situ, and because the DCs containing apoptotic epithelial cells are found in mesenteric lymph (15), we examined whether LP-DCs are unique in their ability to take up apoptotic IECs by observing isolated LP-DCs by transmission electron microscopy. As shown in Fig. 4*D*, the cytoplasm of the R1 cells contained numerous inclusions of 1–1.5 μm in diameter with membranous materials inside them, suggesting that these cells have a high phagocytic activity for apoptotic cells. The cytoplasm of the R2 subset contained fewer, but nonetheless distinct, phagocytic vesicles, a conspicuous Golgi network, well-developed rough endoplasmic reticulum, and numerous round mitochondria, suggesting that R2 is less phagocytic, but more active in protein synthesis and secretion, than R1. Both subsets showed a few finger-like protrusions from the cell body, with the protrusions from R2 cells being finer than those from R1 cells. These observations indicate that LP-DCs are highly active in the metabolism and phagocytosis of apoptotic cells.

Consistent with the above findings, *in vitro* analysis showed that both LP-DC subsets were highly phagocytic, efficiently and vigorously taking up CFSE-labeled apoptotic IECs (Fig. 4*E*), with the R1 subset taking up these cells more avidly than the R2 subset, as evidenced by conspicuous IEC-associated fluorescence staining in the cytoplasm (Fig. 4*F*). PP-DCs were heterogeneous in their phagocytic activity, with the CD11b⁻ fraction of the population taking up apoptotic cells efficiently, and the CD11b⁺ population showing less activity. In contrast, the MLN-DCs had little phagocytic activity regardless of their CD11b expression, and CD11b⁻ SP-DCs showed intermediate levels of phagocytosis.

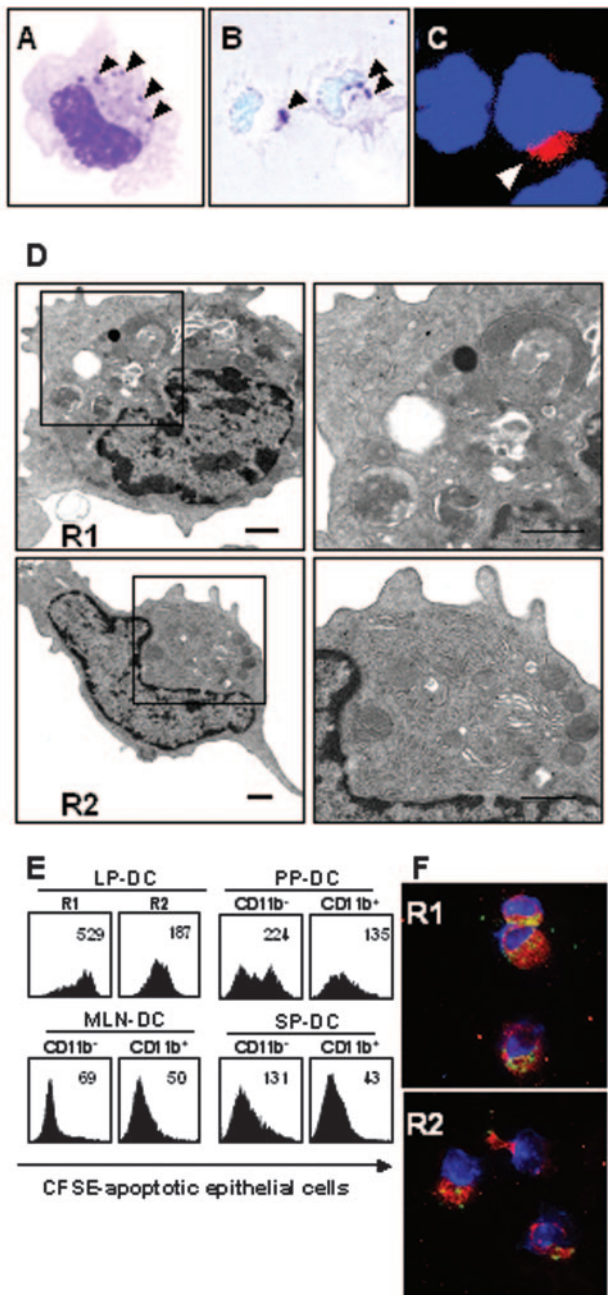


FIGURE 4. LP-DC subsets efficiently endocytose apoptotic IECs. *A–C*, The cytoplasm of MLN-DCs bearing the unique LP-DC phenotype contains cellular debris and fragmented DNA. *A*, FACS-sorted CD11c^{high}CD8 α ^{int} α _L^{low} β ₇^{high} MLN-DC stained with May-Grunwald-Giemsa shows abundant cellular debris in the cytoplasm. *B*, Two CD11c^{high}CD8 α ^{int} α _L^{low} β ₇^{high} MLN-DCs show TUNEL-positive inclusions. *C*, A CD11c^{high}CD8 α ^{int} α _L^{low} β ₇^{high} MLN-DC shows alkaline phosphatase-positive inclusions in the cytoplasm. *D*, Representative transmission electron microscopic views of cells from the LP-DC R1 (*upper*) and R2 (*lower*) subsets. All bars = 1 μ m. *E*, Enriched DCs were cocultured with CFSE-labeled apoptotic IECs for 4 h. The DCs were then collected and stained for CD11c and CD11b and analyzed by flow cytometry. The numbers in the histograms indicate the MFI. *F*, Confocal microscopic images showing the uptake of CFSE-labeled apoptotic IECs by LP-DCs.

LP-DCs presenting IEC-associated Ag induce the differentiation of IL-10- and IL-4-producing CD4⁺ T cells

We next investigated whether LP-DCs could present Ag associated with apoptotic cells to T cells. For this purpose, freshly isolated

IECs from the small intestine were intracellularly loaded with OVA by osmotic shock and then allowed to undergo apoptosis spontaneously. Subsequently, the OVA-loaded apoptotic IECs were cocultured with DCs for 4 h, and the Ag-pulsed DC subsets were subjected to cell sorting. The purified DCs were then cocultured with DO11.10 T cells for 6 days, and T cell proliferation was measured by CFSE dilution. As shown in Fig. 5*A*, both the LP-DC subsets induced vigorous T cell proliferation, whereas a subset of SP-DCs, CD11b⁻ SP-DCs, which displayed moderate phagocytic activity, induced slightly less T cell proliferation than the LP-DCs. The CD11b⁺ SP-DCs, which had much less phagocytic activity than the LP-DCs, induced only weak proliferation. These results show that LP-DCs can take up apoptotic cells and present cell-associated Ags to T cells more potently than splenic DCs.

Mucosal DCs from the PPs and lungs tend to induce Th2 responses in T cell priming assays (3, 32). We therefore addressed whether LP-DCs also show this tendency by examining the cytokine secretion of OVA TCR-transgenic CD4⁺ T cells primed by LP-DCs that had been exposed to OVA-loaded apoptotic IECs. As shown in Fig. 5*B*, T cells primed by LP-DCs expressed significantly higher levels of IL-4 and IL-10 than did SP-DC-primed T

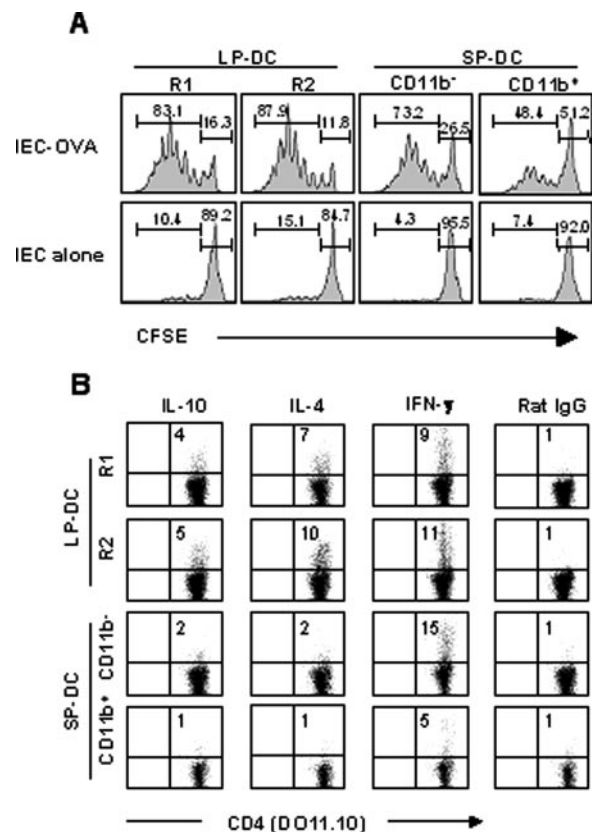


FIGURE 5. LP-DC subsets present IEC-associated Ags. *A*, LP-DC subsets induced cell-associated, Ag-specific T cell proliferation. Freshly isolated IECs were intracellularly loaded with OVA by osmotic shock, then allowed to undergo apoptosis spontaneously. LP-DCs were cocultured with the apoptotic IECs for 4 h, and the DCs were sorted using a FACS Vantage SE. Subsequently, purified DCs bearing the OVA-containing IECs were cultured with naive DO11.10 T cells for 6 days. *B*, The LP-DCs primed DO11.10 T cells to produce IL-4 and IL-10. DCs bearing OVA-containing IECs were then cultured with DO11.10 T cells for 14 days (two rounds of stimulation with a 7-day interval). For intracellular cytokine staining, the T cells were restimulated with the anti-CD3 ϵ mAb for 6 h in the presence of monensin. The data are representative of three independent experiments. Numbers within a quadrant indicate the percentage of cytokine-producing cells.

cells. The production of IFN- γ was comparable among the T cells primed with the different DCs. These results indicate that LP-DCs have a propensity to induce IL-4- and IL-10-producing T cells when exposed to Ag associated with apoptotic IECs.

Discussion

In this study we showed that there are at least two LP-DC subsets in the intestinal LP of unperturbed mice, and they both require CCR7 for their constitutive migration to MLNs. As speculated previously (15), LP-DCs can, in fact, present Ag associated with apoptotic IECs to naive CD4⁺ T cells to induce the differentiation of IL-4- and IL-10-producing T cells. These observations indicate that LP-DCs with unique immunomodulatory activities migrate to MLNs in a CCR7-dependent manner to engage in the presentation of IEC-associated Ags from apoptotic IECs that were phagocytosed locally; this may help explain how a noninflammatory immune response can be maintained in MLNs under steady-state conditions.

Although it is generally accepted that DC migration is largely under the control of chemokines and chemokine receptors, studies of the molecules responsible for the migration of LP-DCs to MLNs in the steady state are lacking. The almost complete absence of DCs bearing the LP-DC phenotype (CD11c^{high}CD8 α ^{int} β ₇^{high} and CD11c^{high}CD8 α ⁻ β ₇^{high}) in the MLNs of CCR7-deficient mice and their marked reduction in the MLNs of *plt/plt* mice, as shown in this study, provide strong evidence that CCR7 is critically important for LP-DC migration to the MLNs. Our unpublished observation that LP-DCs were found in the intestines of CCR7-deficient mice also supports this hypothesis. The ligands for CCR7 are CCL19 and CCL21. The *plt/plt* mouse strain congenitally lacks the expression of CCL19 and CCL21-Ser, but does express another CCL21 gene product, CCL21-Leu, in lymphatic endothelial cells (20). Thus, our data point to a critical role for CCR7-mediated signaling at the step of DC entry into the intestinal lymphatics, although additional investigation is required to pinpoint the site(s) of action of CCR7-mediated signaling. A pivotal role for CCR7 in the migration of skin DCs to afferent dermal lymphatics has been indicated in a report by Ohl et al. (23).

CCR7-mediated signaling may also play a role in the maturation and survival of LP-DCs transported to the MLNs, because CCR7 has been shown to induce antiapoptotic signaling (33) and terminal activation (34) in DCs. Although this issue could, in theory, be investigated by comparing the fate of CCR7-deficient vs wild-type LP-DCs within MLNs upon their injection into the intestinal lymphatics, a technical difficulty involved in applying this procedure to the mouse has prevented us from performing such experiments.

It is of note that although LP-DCs appeared to be relatively immature, showing a CD80^{low}CD86^{int}MHC class II^{low}CD40^{low} phenotype, they expressed CCR7 and functionally responded avidly to CCL21 like mature DCs (19, 35). Given that immature DCs, albeit CCR7 negative initially, up-regulate their CCR7 expression upon ingestion of opsonized apoptotic cells (36), it is tempting to speculate that LP-DCs acquire CCR7 expression through the ingestion of apoptotic IECs *in situ*.

In contrast to the LP-derived DCs, the number of PP-derived DCs (CD8 α ^{high} β ₇^{int} and CD8 α ⁻ β ₇^{low}) was not lower in the MLNs of *plt/plt* and CCR7-deficient mice (Fig. 4), indicating that PP-DCs do not depend on CCR7 signaling for migration to the MLNs. Previous studies showed that different PP-DC subsets differentially express multiple chemokine receptors, including CCR1, CCR2, CCR5, CCR7, CCR9, and CCR10, with all the subsets expressing functional CCR7 (4, 37). Although PP-DCs may use CCR7-mediated signaling for their localization within PP microdomains, as suggested previously (4), our study indicates that PP-DCs depend

on non-CCR7 ligand chemokine(s) for their steady-state migration to MLNs.

Although recent studies of murine LP-DCs have identified several distinct subsets, i.e., CD11c⁺CD8 α ⁻CD11b⁺ (14, 38), CD11c⁺CD8 α ⁻CD11b⁻ (11, 14), CD11c⁺CD8 α ⁺CD11b⁻, CD11c^{int}CD8 α ⁻CD11b⁺B220⁺ (14), and CD11c⁻CD11b⁻ (30), our data presented in this paper clearly showed that, among these groups, the major contributors are from two populations, i.e., CD11c^{high}CD8 α ^{int}CD11b^{low} α _L^{low} β ₇^{high} (R1) and CD11c^{high}CD8 α ⁻CD11b^{high} α _L^{low} β ₇^{high} (R2), in unperturbed mice. We also observed CD11c^{int}CD11b^{high} cells to be abundant in the LP, but we did not include them in our current analysis, because they appeared to be different from conventional DCs, with numerous cytoplasmic eosinophilic granules. There were also low numbers of DC-like cells in our LP cell population, but they constituted only a very small proportion of the cells compared with R1 and R2, and thus they were not analyzed in the current study.

The recently reported CD11c⁺CD8 α ⁻CD11b⁻ LP-DCs constitutively expressing the IL-12 p40 promoter (11) and APCs with the CD11c⁻CD11b⁻ phenotype that constitutively express CD70 (30) may have been among the cell populations we did not study. In addition, a recent study identified CX3CR1⁺ LP-DCs that form dendrites through the IECs of the terminal ileum to sample bacteria in the lumen directly (10). Because these CX3CR1⁺ LP-DCs are CD11c^{high}CD11b^{high}, they resemble the R2 subset of our analysis. However, our preliminary observation indicates that hardly any cells in the R2 subset were reactive with an anti-CX₃CR1 mAb (M. H. Jang and N. Sougawa, unpublished observation).

Previous reports by Huang et al. (15) using mesenteric lymphadenectomized rats showed a DC subset in the intestinal lymph that constitutively transports apoptotic IECs to the T cell areas in MLNs. Our study demonstrated that although MLN-DCs with the LP-DC phenotype are poorly phagocytic, there is irrefutable evidence of previous phagocytosis, inasmuch as they contain much intracytoplasmic cellular debris, and both CD8 α ^{int} and CD8 α ⁻ LP-DC subsets can ingest apoptotic IECs vigorously and present IEC-associated Ag to CD4⁺ T cells *in vitro*. These results strongly indicate that MLN-DCs containing considerable cellular debris correspond to the cells identified in the intestinal lymph by Huang et al. (15), and that the site of apoptotic cell acquisition by these cells is the LP.

Our observation that LP-DCs could polarize CD4⁺ T cell differentiation to favor IL-4- and IL-10-producing T cells subsequent to coculture with Ag-loaded apoptotic IECs parallels the observation by Iwasaki and Kelsall (3) that PP-DCs generate T cells that produce high levels of IL-4 and IL-10 and less IFN- γ , as well as the observation by Alpan et al. (39) that Ag-loaded DCs present in MLNs can induce T cells to produce IL-4 and IL-10. Thus, certain intestinal DCs, including the LP-DCs, may have a tendency to induce Th2, rather than Th1, cells, which may be, at least in part, related to their localization to a specific microenvironment. Because phagocytosis of apoptotic cells has been reported to result in an anti-inflammatory state via transcriptional suppression of IL-12 (40), DCs that are closely colocalized with IECs in the LP may become locally imprinted not to induce Th1 cells upon ingestion of apoptotic IECs. In addition, conditioned medium of IECs have been shown to induce DCs to release IL-6 and to prime Th2 responses (41). Thus, the local microenvironment appears to confer on DCs a propensity to induce Th2 cells.

Collectively, these data provide strong evidence that CCR7 is critical for the recruitment of LP-DCs into the MLNs under steady-state conditions. Because these LP-DCs phagocytose apoptotic IECs and present IEC-associated Ag to induce IL-4- and IL-10-producing CD4⁺ T cells, they are likely to be involved in the

immunomodulation of T cells within the MLNs. Thus, our study strengthens the idea that CCR7 is involved in the maintenance of peripheral tolerance (23) and points to a role for CCR7 in the recruitment of immunomodulatory DCs to the MLNs.

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Disclosures

The authors have no financial conflict of interest.

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