CCR7 Essentially Contributes to the Homing of Plasmacytoid Dendritic Cells to Lymph Nodes under Steady-State As Well As Inflammatory Conditions

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The chemokine receptor CCR7 represents an important determinant for circulating lymphocytes to enter lymph node (LN) via high endothelial venules. High endothelial venules also represent the major site of entry for plasmacytoid dendritic cells (pDC). In the steady-state, murine pDC have been suggested to home to LN engaging the chemokine receptors CXCR3, CXCR4, and CCR5, whereas responsiveness to CCR7 ligands is thought to be acquired only upon activation. In this study, we show that already resting pDC express minute amounts of CCR7 that suffice to trigger migration to CCL19/CCL21 in vitro. Upon activation with TLR ligands, CCR7 levels on pDC are strongly increased. Notably, CCR7-deficient mice display substantially reduced pDC counts in LN but not in bone marrow and spleen. Adoptive cell transfer experiments revealed that under both steady-state as well as inflammatory conditions, the homing of CCR7-deficient pDC is severely impaired, indicating that the reduced cell counts of naive pDC observed in CCR7−/− mice reflect an intrinsic homing defect of pDC. Together, these observations provide strong evidence that similar to naive lymphocytes, nonstimulated pDC exploit CCR7 to gain entry into LN. This adds to the repertoire of chemokine receptors permitting them to enter diverse tissues. *The Journal of Immunology*, 2011, 186: 3364–3372.

T he chemokine receptor CCR7 has been shown to confer distinct migratory properties to leukocytes in both homeostatic and inflammatory conditions. CCR7 expression on recirculating lymphocytes represents a key factor for lymph node (LN) entry via high endothelial venules (HEV). Consequently, CCR7-deficient mice display severely reduced LN cellularity and disturbed cellular composition owing to impaired homing of naive T cells and to a lower extent than of B cells (1). Furthermore, the LN architecture is severely disturbed in these mice, as CCR7, in combination with CXCR5, contributes to the segregation of T zone and B cell follicles (1, 2). Additionally, the mobilization of tissue-resident myeloid dendritic cells (mDC) requires CCR7 (3). Although immature mDC do not express this receptor, CCR7 is induced upon maturation to semimature, tolerogenic, as well as to mature mDC. CCR7-expressing mDC are attracted by CCL21 present on lymphatic vessels and allow subsequent migration toward the draining LN via the afferent lymphatics. The importance for CCR7 expression on DC was shown by the complete failure of oral as well as respiratory tolerance induction in CCR7-deficient mice due to the lack of cellular Ag transport into the mesenteric and bronchial LN, respectively (4–6).

Plasmacytoid DC (pDC) represent a distinct population of DC. Originally defined as CD11c+CD11b+low/B220+Ly6c+ cells, at least two more surface markers are available to detect murine pDC. Whereas the mAb clones 120G8 and PDCA-1 are suitable to identify pDC, their cognate Ag BST-2 is induced upon activation on a variety of cells such as mDC and some B cells. In contrast, expression of Siglec-H seems to be restricted to pDC. Thus, a combination of markers is available that can be used to discriminate more accurately pDC from other cell subsets. Although pDC share some features with mDC, such as expression of CD11c and MHC class II, the ability to cross-present Ag, and to induce T cell based immunity as well as tolerance, both cell types still appear to be quite different from each other with regard to diverse criteria such as morphology and physiologic distribution (7, 8). Except for the intestine, pDC are rarely found in peripheral tissues and do not enter the draining LN via the afferent lymphatics following activation (9). pDC have been reported to migrate to sites of inflammation and to infiltrate tumors as well as solid organ transplants (10–12). In these scenarios pDC again are not found within the lymph fluid but are thought to directly re-enter the blood before homing to lymphoid organs (12). Thus, transport via the blood stream seems to be a rather exclusive route for pDC traffic in both steady-state and inflammatory conditions, and extravasation into LN through HEV has been postulated to reflect the primary, if not sole, mechanism for entry (12). Indeed, pDC have been observed to tightly interact with HEV, although the subsequent transmigration step is less efficient than in T cells (13). On the molecular level, pDC engage similar mechanisms to accomplish LN entry as T cells, including rolling mediated by CD62L and integrin-dependent firm adhesion. However, in contrast to T cells that increase integrin affinity by CCR7-mediated signaling, pDC were reported to use other chemokine receptors such as CXCR3, CXCR4, CCR5, CCR10, and ChemR23 (7, 14–17).
However, the exact contribution or concerted function of either of them remains controversial. A possible contribution of CCR7 to the LN homing of pDC in mice has been considered unlikely since it was demonstrated in several reports that murine pDC are CCR7 negative or low, and functionally were considered unresponsive to CCR7 ligands (15, 18, 19). In contrast to murine pDC, there are conflicting results regarding CCR7 expression on human pDC. Although evidence exists suggesting high levels of CCR7 on blood pDC (14, 20), yet without conferring any responsiveness toward CCR7 ligands, others observed only low levels of CCR7 on resting pDC (16, 21, 22). Another feature possibly relevant for pDC migration that is not conserved between men and mice is represented by the receptor specificity for CCL21. In mice but not humans this chemokine has been reported to also bind to and trigger CXCR3, thereby potentially contributing to pDC migration (23, 24). Along the same line, human but not murine pDC express ChemR23, a chemotactically functional receptor present on all blood-borne pDC. The corresponding ligand, chemerin, is expressed on the luminal side of the HEV, and as a result, this receptor–ligand interaction also essentially contributes to LN homing of pDC in humans (17). pDC from both species upregulate CCR7 upon stimulation with TLR ligands, a finding that closely resembles the activation profile of mDC (3, 14).

In the current study we present a protocol that allows for a highly efficient differentiation of pDC from bone marrow (BM) cells in vitro. Using these cells as well as ex vivo-derived pDC we show that already nonstimulated, naïve pDC express CCR7. Adoptive cell transfers reveal that CCR7-deficient pDC shown impaired homing to resting as well as inflamed LN. These results help explain why we observed strongly reduced numbers of pDC residing in LN of CCR7-deficient mice. Collectively, this study identifies CCR7 as an important LN homing receptor for pDC under both steady-state and inflammatory conditions.

Materials and Methods

Mice

Wild-type C57BL/6, C57BL/6-CCR7<sup>−/−</sup>, C57BL/6-Ly5.1, and BALB/c-CCR7 mice were bred in the animal facility of Hannover Medical School. BALB/c and C57BL/6 wild-type mice were additionally purchased from Charles River Laboratories (Sulzfeld, Germany). All experiments have been approved by the Institutional Review Board and the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelei-Isicherheit.

Antibodies

For flow cytometry the following mAbs were used: 120G8-Alexa 488 (Acris), Siglec-H-bio (clone 440c; Hyctl Biotechnology), B220-PerCP-Cy5.5, CD11b-PE-Cy7, CXCRC3-PE, CCR7-PE, CD86-allophycocyanin (all from eBioscience), Ly5.1-allophycocyanin, CD62L-allophycocyanin (all from BioLegend), CD11a-FITC (Sigma-Aldrich), CD18-FITC (Caltag Laboratories). B220-Pacific Orange (clone RA3-3A1) and CD4-PO (clone RnCd4-2) were grown and labeled in our laboratories. In some cases CCR7 was detected by binding of recombinant ELC-Ig fusion protein (25) followed by anti-ß-hlg-Fc-PE (Jackson Immunological Research Laboratories). Secondary conjugates streptavidin-Cy5 (Caltag Laboratories), streptavidin-PerCP (BD), or streptavidin-Pacific Orange (Invitrogen) were applied to detect biotinylated primary Abs.

Cell preparation and flow cytometry

Single-cell suspensions of various lymphoid organs were obtained by mincing the tissue through a nylon mesh. Blood samples were treated with citric acid to prevent clotting. Bone marrow was flushed out of femur and tibia. The intraepithelial lymphocyte (IEL) fraction of the small intestine was prepared according to standard protocols using EDTA for detachment (26). Liver leukocytes were obtained according to a modified protocol based on previously described methods (27, 28). In brief, after perfusion livers were cut into small pieces, then incubated in RPMI 1640 medium supplemented with 10% FCS, 25 mM HEPES, 0.25 mg/ml collagenase IV, and 12.5 μg/ml DNase I (Sigma-Aldrich) for 45 min at 37°C. Enzymatic activity was terminated by addition of EDTA to a final concentration of 20 mM. The medium was then filtered through a nylon mesh. Leukocytes were recovered from intestinal and liver samples by density gradient centrifugation on Percoll (Amersham Biosciences) as described before (26). Samples from spleen (SPL), blood, liver, and BM were subjected to erythrocyte removal by treatment with hypotonic NH4Cl. Staining of the leukocytes was performed in PBS±i B5 without calcium and magnesium containing 2% FCS on ice after initial blocking with rat serum except for anti-CCR7, which requires staining at 37°C. For detection of CCR7 using the ELC-Ig fusion protein, the cells were blocked before the protein was applied. The human IgG portion was detected using anti-human IgG Abs preadsorbed with rat serum and mouse serum. Additional Abs were applied in subsequent steps only. All samples were supplemented with DAPI to exclude dead cells from the analysis. Flow cytometry was performed on an LSRII flow cytometer (BD Biosciences); the data were analyzed using WinList 5.0 excluding dead cells and cell doublets.

Generation of BM-derived pDC

Cell culture supernatant containing Flt-3L was harvested from high-density spinner cultures of the B16FL tumor cells (29) twice a week. The Flt-3L concentration within the conditioned medium was determined by ELISA and was found in a range of 150–300 ng/ml.

For the differentiation of BM-derived pDC (BMPDC), 2 × 10<sup>6</sup> total C57BL/6 BM cells per milliliter were cultured for 8 d in RPMI 1640 medium supplemented with 10% FCS, pyruvate, glutamine, penicillin/streptomycin, gentamicin (all purchased from Invitrogen), and conditioned B16FL tumor cell medium to a Flt-3L final concentration of 8–15 ng/ml. The conditioned supernatant contributed a proportion of <6% (±v/v) of the total culturing medium for the BM cells. Alternatively, purified recombinant Flt-3L derived from a mouse myeloma cell line (R&D Systems) was tested at concentrations up to 100 ng/ml. Medium was replaced once after 4 d of culturing. For maturation of the pDC, medium was replaced by Flt-3L-free medium containing additionally 2 μg/ml R837 or R848 (InvivoGen) for 18 h.

In vivo expansion of pDC

C57BL/6 wild-type or CCR7<sup>−/−</sup> mice were s.c. transplanted 1 million B16FL tumor cells per flank as described previously (29). Tumor growth was monitored over a period of 10–14 d until sacrifice. For in vitro migration assays, pDC were enriched from pooled LN and SPL using the pDC isolation kit 2 (Miltenyi Biotec).

Adaptive cell transfer experiments

For adoptive transfers, cell preparations were differentially labeled with the fluorochromes TAMRA and DDAO (Invitrogen) according to a protocol described before (30) employing a final concentration of 10 μM TAMRA and 0.5 μM DDAO. Two to 4 million of each pDC type were mixed at a ratio of ~1:1 and transferred i.v. Fluorescent pDC were recovered after the indicated times and analyzed by flow cytometry. For testing inflammatory conditions mice were s.c. injected with 0.25 μg cholera toxin (Sig- ma-Aldrich) in both flanks 1 d before adoptive transfer of cells.

RNA preparation and real-time PCR

In vivo-expanded pDC were prepared from pooled LN of wild-type (WT) or CCR7-deficient mice. pDC were enriched using the pDC isolation kit 2 (Miltenyi Biotec), supplemented with an additional biotinylated Ab against CD11b (clone M1/70, grown and labeled in our laboratories). The cells were then stained for sorting. pDC were addressed as Siglec-H<sup>+</sup>B220<sup>+</sup> CD11b<sup>+</sup>CD123<sup>+</sup>DAPI<sup>−</sup> cells, and sort purity was reanalyzed to ensure >97% purity for pDC in the collected fraction. After sorting, the cells were directly subjected to RNA preparation using the Absolutely RNA MicroPrep Kit (Stratagene). RNA was reverse transcribed (SuperScript II; Invitrogen) and real-time PCR was performed in duplicates on the recovered cDNA samples using the SYBR Premix Ex Taq kit (Takara Bio). Relative comparative quantification of CCR7 expression levels was done based on GAPDH expression. The primers used were: mCCR7F<sub>RT</sub> 978, 5′-CCAGCAAGCAGCTCAA-CCG-3′, mCCR7R<sub>RT</sub> 1069, 5′-GCCGATGAAGGCATACAAGA-3′, GAPDH forward, 5′-TGCCACCCCAACTCCTTGAG-3′, GAPDH reverse, 5′-GGATGCCAGGATGATGGTC-3′.

Transwell migration assay

Transwell inserts (6.5 mm diameter) with 5-μm membrane pores were coated with collagen IV overnight as described previously (31). One hundred microliters of a cell suspension containing a 1:1 ratio of WT/
Ly5.1⁻ and CCR7⁻ /Ly5.1⁺ BM-derived (BMpDC) or in vivo-expanded, MACS-enriched pDC was applied per transwell to the upper chamber. Migration was tested toward the chemokines CCL19 and CCL21 present in the lower compartment in a total volume of 400 μl medium. Migrated cells were harvested after 3 h incubation at 37˚C and subjected to analysis by flow cytometry. For cell enumeration, a defined number of Fluoresbrite beads for detection also by flow cytometry were added. The migration index (MI) was calculated according to the formula: MI = no. cells_{sample}/no. cells_{control}.

Statistical analysis

All statistical analyses were performed using the unpaired Student t test. The p values are given within the diagrams.

Results

Reduced numbers of pDC in LN of CCR7-deficient mice

Analyzing the frequencies of lymphocyte populations in various lymphoid organs and compartments in WT and CCR7-deficient C57BL/6 mice, we observed strikingly reduced proportions of pDC. Defined by 120G8⁺B220⁺ marker expression, pDC frequencies in peripheral LN (PLN), mesenteric LN (MLN), and blood of CCR7⁻ / mice were significantly lower whereas there were no such differences evident in SPL, liver, BM, and the IEL compartment of the small intestine (Fig. 1A). In absolute cell numbers, the differences between WT and CCR7-deficient mice regarding pDC numbers in PLN and MLN are even more pronounced, whereas neither pDC frequencies nor total cell counts in BM of CCR7-deficient mice are altered. This observation is reminiscent to those made for T cells, except that peripheral blood T cell counts are not affected by CCR7 deficiency (Fig. 1B, 1C). A similarly strong reduction of pDC numbers was found in CCR7-deficient mice on a BALB/c background although overall higher frequencies of pDC occur in this mouse strain (not shown).

Because the LN architecture is severely disturbed in CCR7-deficient mice (1), we sought to determine the localization of pDC within the LN microdomains (Supplemental Fig. 1). In WT LN, most pDC can be detected in the T zone, preferentially in regions adjacent to B cell follicles. However, pDC appear to be completely excluded from the B zone (Supplemental Fig. 1A, 1C). In the LN of CCR7-deficient mice, only very few pDC were detectable, but those identified were still excluded from the B cell follicles (Supplemental Fig. 1B, 1D).

Resting pDC express CCR7

CCR7 expression on murine pDC has been reported to only occur following activation of these cells (14). Because the reduced numbers of pDC were observed in mice under noninflammatory, so-called steady-state conditions, we addressed the question whether resting pDC already express CCR7. Flow cytometry on pDC, B cells, and T cells was performed using freshly prepared LN-derived lymphocytes, and the signals elicited by the Ab were validated by including isotype control as well as Ab staining on parallel sets of CCR7-deficient lymphocytes. Whereas substantial CCR7 expression can be detected on WT B and T cells, pDC express only minute yet distinctive amounts of this chemokine receptor. Summarizing all experiments, we consistently found a CCR7-specific staining when compared with isotype control on WT pDC, whereas specific signals could never be detected on pDC derived from CCR7-deficient mice (Fig. 2A). As all further studies were performed using either in vitro-generated pDC (see below) or in vivo-expanded pDC, we included these populations in the expression analysis of CCR7. As expected, the CCR7 expression levels detected in both cell types were very similar to ex vivo-derived pDC (Fig. 2B). To correlate the flow cytometry data with an alternative detection method, we isolated in vivo-expanded pDC from LN of WT and CCR7-deficient mice and subjected these cells to real-time PCR analysis. A CCR7-specific amplification occurred only in WT samples. The specific CCR7 signals were correlated to GAPDH as a housekeeping gene (Fig. 2C) for quantification.
To rule out the possibility that the expression of other LN homing molecules is affected by CCR7 deficiency, we stained LN-derived pDC for CD11a, CD18, CXCR3, and CD62L. Comparative expression analyses were performed on WT and CCR7-deficient leukocytes, including evaluation of expression levels on pDC, B cells, and T cells (Supplemental Fig. 2A). Of interest, we failed to detect differences in the expression levels of any of the molecules on the cell populations analyzed. We therefore conclude that lack of CCR7 is likely to be the direct cause for the reduced numbers of resting pDC found in LN of CCR7-deficient mice.

Differentiation of pDC with high efficiency in vitro

For further studies addressing the migration of CCR7-deficient pDC compared with WT in vitro as well as in vivo, the numbers of naturally occurring pDC are too limited. Therefore, we exploited two different methods to gain access to suitable numbers of these cells. First, we expanded pDC in vivo by implanting B16FL melanomas, as published elsewhere (29). These tumor cells secrete Flt-3L, which fosters the expansion and differentiation of pDC. Ten to 14 d later the leukocyte population of treated mice contained up to 15% pDC (not shown). As an alternative, the in vitro differentiation of pDC from BM using recombinant Flt-3L had been reported (15). Although high concentrations of Flt-3L (200 ng/ml) were applied in this study, the in vitro differentiation was rather inefficient in both cell yield and purity (15). We therefore tested serial dilutions of the cell culture supernatant of the Flt-3L–producing tumor cell line B16FL (supFlt-3L) directly for the in vitro differentiation of pDC (see also Materials and Methods for details). BMpDC differentiated by supFlt-3L show the expected phenotype, as they are 120G8'B220'Siglec-H'. Expression of CD11b is moderate and approximately a magnitude below the mDC that differentiate in parallel within the bulk cultures (Fig. 3A). Titration assays revealed that the supFlt-3L is much more potent in driving both pDC differentiation and cell expansion compared with commercially available rFlt-3L. Optimal purity of >50% BMpDC and high cell expansion were observed at concentrations of 5–15 ng/ml supFlt-3L. In marked contrast, using the rFlt-3L, the best BMpDC purity of <40% was obtained using as much as 100 ng/ml rFlt-3L but still at poor rates of cell expansion (Fig. 3B). BMpDC in the bulk culture obtained by coculturing with either ligand display a resting phenotype defined by low CD86 expression and hardly detectable surface CCR7. However, myeloid DC contained in the same culture display a CD86↑CCR7↓ phenotype (Fig. 3C, upper panel). Upon stimulation with the TLR7 ligand R837, BMpDC upregulated both CD86 and CCR7 to intermediate expression levels (Fig. 3C, lower panel). These results establish that significantly more pDC can be obtained via the protocol presented above. It is currently unknown why commercially available Flt-3L is inferior in its capacity to generate pDC. We assume that bypassing any purification steps preserves the potency of the Flt-3L, as both are derived from eukaryotic sources and should therefore share high similarity.

Resting pDC migrate to CCR7 ligands in vitro

Resting pDC express comparably low levels of CCR7. To demonstrate that minute amounts of CCR7 as observed are sufficient to direct pDC chemotaxis, we performed transwell migration assays testing the responsiveness of WT and CCR7-deficient pDC to the CCR7 ligands CCL19 and CCL21 (Fig. 4). We also analyzed migration toward CCL25, the ligand for CCR9, known to induce strong chemotaxis of pDC (9). Migration of nonactivated WT BMpDC toward both CCL19 and CCL21 was rather inefficient with MI of 1.426±0.227 for CCL19 and 1.988±0.431 for CCL21 (Fig. 4A). Interestingly, although being unresponsive to CCL19, CCR7-deficient BMpDC migrate moderately toward CCL21 (MI = 1.501 ± 0.188), a finding that is in line with previous reports suggesting CXCR3 as an alternative receptor for CCL21 (23). However, WT BMpDC migrated significantly better toward both chemokines than did their CCR7-deficient counterparts. In contrast, both populations migrated equally well toward CCL25 (MI = 2.953 ± 1.667 and 2.978 ± 1.617), indicating that CCR7 deficiency does not impair the migratory capacity of the cells (Fig. 4A). As BMpDC may display a migration behavior distinct from ex vivo-derived pDC, we isolated in vivo-expanded pDC from...
pooled LN by MACS isolation and subjected them to transwell migration assays (Fig. 4D). Whereas we observed even better migration toward CCL25 when compared with BmpDC, migration toward CCR7 ligands was rather limited under these experimental conditions. Poor migration was observed toward CCL19 without differences between WT and CCR7-deficient cells. However, migration toward CCL21 was clearly detectable with a strong yet not significantly better migration of WT than CCR7-deficient pDC (MI = 1.457 ± 0.209 and 1.288 ± 0.191, respectively).

Adoptive transfer to study migration of resting pDC in vivo

To test the impact of CCR7 expression on pDC migration in a more physiological context than is represented by the in vitro transwell migration assays, we chose to test both in vivo-expanded and in vitro-differentiated pDC for competitive homing in vivo in WT recipient mice (Fig. 5). Therefore, equal numbers of WT and CCR7-deficient pDC were differentially labeled with either TAMRA or DDAO, mixed at a ratio of 1:1, and then adoptively transferred i.v. into the recipients. Determining the numbers of transferred cells in the lymphoid organs, we found that in vivo-expanded CCR7-deficient and WT pDC homed at similar frequencies to BM and SPL. In contrast, CCR7-deficient pDC showed an ~50% reduction in their homing capacity to LN when compared with WT cells (Fig. 5A). However, note that CCR7 deficiency had a stronger impact on LN homing of other cells that were adoptively transferred along with the pDC such as B220⁺ B cells and 120G8⁻ B220⁺ cells, which primarily consist of T cells (Fig. 5B, 5C). In line with the results from the in vivo-expanded pDC, the LN homing of the in vitro-generated BmpDC is also reduced in CCR7-deficient cells in the same experimental setup (Fig. 5D). These findings strongly support the hypothesis that resting pDC directly rely on the expression of CCR7 to efficiently home to LN.

**FIGURE 3.** Generation of BmpDC driven by cell culture supernatant of Flt-3L–secreting B16FL tumor cells. A, C57BL/6 BM cells were cultured for 8 d in medium supplemented with conditioned medium derived from Flt-3L–secreting B16FL tumor cells at a final concentration of 5 ng/ml Flt-3L. FACS analysis is shown of cells using the Abs indicated. Cells displaying pDC characteristics are highlighted by red gates (representative result from three independent experiments). B, Titration of Flt-3L derived from B16FL cell culture supernatant (△) or commercial Flt-3L (■). Cultures were started with 10⁵ BM cells. Given are the purity (left panel) and total yield (right panel) of 120G8⁻B220⁺ BmpDC (mean ± SD; n = 4 independent experiments). C, Activation and CCR7 induction on BmpDC in vitro. C57BL/6 WT and CCR7-deficient BmpDC cultures were harvested on day 8, transferred into fresh full medium, and split. One half was left untreated (upper panel) whereas the other was stimulated with 2 μg/ml R848 (lower panel). After 12 h, BmpDC were addressed as 120G8⁻B220⁺ cells and analyzed for the expression of CD86 and CCR7.

**CCR7-deficient pDC display impaired LN homing during inflammation**

After having elaborated an important role for CCR7 in the homing of immature pDC under steady-state conditions, we additionally sought to determine the impact of CCR7 on LN homing of resting and mature pDC at different scenarios of inflammation. For comparison between resting and activated pDC, we first assessed the migratory potential R848-stimulated BmpDC in vitro in transwell assays. As expected, maturation and subsequent CCR7 induction on BmpDC greatly increased the migration toward CCL19 (MI = 3.356 ± 2.382) and CCL21 (MI = 5.982 ± 4.480) in WT cells. CCR7-deficient BmpDC remained completely unresponsive not only to CCL19 but also to CCL21, thus loosing the residual migration capacity displayed by resting pDC (Fig. 4B).

This finding may support the idea of CXCR3 as an alternative functional receptor for CCL21 on resting pDC (23). As CXCR3 is downregulated upon stimulation (14, 16), CCR7-deficient pDC no longer respond to CCL21 stimuli. Still, both resting as well as activated BmpDC remained less attracted by CCR7 ligands than mature myeloid DC that most efficiently migrated toward CCL19 (MI = 4.186 ± 2.337) and CCL21 (MI = 6.237 ± 4.862; Fig. 4C).

To assess the in vivo migration of activated pDC, WT and CCR7-deficient BmpDC were differentiated and matured in vitro by R848 and then subjected for competitive homing in WT recipient mice that were left untreated prior to cell transfer (Fig. 6A) or s.c. inoculated with cholera toxin to mimic an inflammatory setting in the tissue draining LN (Fig. 6B). Again, we observed a similarly moderate reduction in the homing efficiency of CCR7-deficient activated pDC into steady-state LN as seen for resting pDC, despite the strong induction of CCR7 following stimulation with R848 (Fig. 6A). Furthermore, a similarly impaired homing was observed when analyzing the migration of R848-activated pDC to inflamed LN (Fig. 6B). Finally, we addressed the recruitment of resting pDC into inflamed LN by adoptive transfer of WT and CCR7-deficient pDC into mice s.c. inoculated with cholera toxin, as this scenario probably mimics best the homing of cells under the condition of a peripheral inflammation. Again, we observed a similarly defective homing of CCR7-deficient pDC into the draining as well as the nondraining LN. Homing of pDC into LN represents a rather rare event even if abundant numbers are available in blood, as is the case after the adoptive transfer of 8 × 10⁶ total pDC. In a representative experiment we recovered 44,720 ± 10,790 transfected pDC from SPL, 1,425 ± 437 from MLN, 113 ± 41 per nondraining PLN, and 172 ± 101 from an inflamed...
This demonstrates that pDC easily access the SPL whereas only few cells are able to enter LN through the HEV. The slightly increased numbers of pDC found in draining versus nondraining LN are most likely based on the overall increased LN size due to the inflammation, as the frequencies of pDC in total cells were found to be very similar (not shown).

Altogether, these results provide strong evidence that CCR7 plays an essential role for the homing of pDC to LN under both steady-state and inflammatory conditions.

**Discussion**

The results presented in this study extend the knowledge regarding the role of CCR7 in the migration of pDC, particularly in the context of their homing to LN. While CCR7 expression and function is well documented for activated pDC, we show in this study that resting pDC already express low amounts of functional CCR7 and that this expression substantially contributes to their entry into LN under steady-state as well as under inflammatory conditions. This newly discovered relevance of CCR7 function was observed for pDC regardless of whether they were derived ex vivo or were differentiated in vitro from BM cells. Although the expression level of CCR7 on pDC appears to be comparably low, this level is yet sufficient to trigger migration to CCR7 ligands, CCL19 and CCL21, as shown by the in vitro assays using BmpDC. A weaker migration observed in in vivo-expanded pDC may be caused by handling of the cells during the enrichment and/or reduced responsiveness due to abundant ligand exposition within lymphoid organs they are derived from. However, along with the results obtained from the competitive homing of WT and CCR7-deficient pDC in vivo, these findings provide solid evidence for a substantial involvement of CCR7 in the LN homing of either type of pDC in a physiological environment. This adds new aspects to previous studies that considered mainly CCR7-independent pathways for pDC recruitment into LN under steady-state conditions. In line with their chemokine receptor expression profile, murine immature pDC are enabled to migrate toward ligands for CXCR3, CXCR4, CCR5, and CCR9 (9, 15, 16, 21). CCL25, the ligand for CCR9, is not present on HEV but is critically required for homeostatic entering of the intestinal mucosa (9). CXCL9, CXCL10, and CXCL11, the ligands for the CXCR3, as well as CCL3, CCL4, and CCL5 (which all bind to CCR5), represent inflammation-inducible chemokines that may be present on activated HEV but are not found at these sites under steady-state conditions. Considering the repertoire of chemokines presented by HEV to blood cells under steady-state conditions, CXCL12, the ligand for CXCR4, would be a potential candidate mediating steady-state LN homing of pDC, as this chemokine has been reported to be constitutively expressed HEV. However, stimulation via CXCR4 alone had only a minor impact on the transendothelial migration of pDC (16).

Thus, the array of chemokine receptors known before to be expressed by resting pDC suffered from the reputation that it would

**FIGURE 4.** pDC migrate toward CCR7 ligands in vitro. **A–C, C57BL/6 Ly5.1** and CCR7-deficient Ly5.1 BM cultures containing ~50% BmpDC were harvested at day 9 to yield resting BmpDC or were activated 12 h earlier with 2 μg/ml R848 to obtain activated BmpDC. For transwell migration, 1–2 x 10^6 cells of a mix containing equal numbers of WT and CCR7-deficient BmpDC were applied onto collagen-coated membranes. Chemokines CCL19 (10–30 nM), CCL21 (30 nM), and CCL25 (300 nM) were added to the medium in the lower chambers. After 3 h, cells present in the lower chamber were counted and phenotypically analyzed by flow cytometry. Resting BmpDC were defined as 12G8^+^B220^-^ cells while activated BmpDC were detected as 12G8^+^B220^-^Siglec-H^-^CD11b^int^ cells and were found to have a mature CD86^+^ phenotype (not shown). WT cells were discriminated from CCR7-deficient cells by expression of Ly5.1. Shown are migration indices toward CCL19 and CCL21 of resting BmpDC (A) or R848-stimulated BmpDC (B) as well as mature mDC (C). For resting BmpDC and mature mDC, the migration toward CCL25 is shown as well. Depicted are results from at least two independent experiments performed in triplicates (dots represent individual transwells; bars indicate mean values). **D, In vivo-expanded pDC from Ly5.1 WT and Ly5.1 CCR7-deficient mice were obtained from LN and SPL using negative isolation by MACS.** pDC enriched to a purity of ~80% were used for transwell assays analogous to the situation described for **A.** Shown are the results from two independent experiments (dots represent individual transwells; bars indicate mean values).
not meet the requirements for efficient homeostatic homing into LN. Instead, this receptor signature appeared suitable for pDC recruitment into peripheral organs and sites of inflammation. This view is now more balanced by adding CCR7 to the list of chemokine receptors available in resting pDC for homeostatic LN trafficking. Consequently, it may not be surprising that CCR7-deficient mice display a pronounced phenotype when comparing the absolute numbers but also the frequencies of pDC in PLN as

**FIGURE 5.** Impaired homing of CCR7-deficient pDC following adoptive transfer. For adoptive transfer, WT and CCR7-deficient cells were split into two halves, which were labeled with TAMRA or DDAO. DDAO-labeled WT cells were mixed with TAMRA-labeled CCR7-deficient cells at a 1:1 ratio. In cross-labeling experiments, TAMRA-labeled WT and DDAO-labeled CCR7-deficient cells were used. Mixtures of pDC were i.v. injected into C57BL/6 recipient mice, and homing of cells to SPL, PLN, MLN, and BM was analyzed 18 h later. The ratio of CCR7-deficient versus WT cells was determined and shown as the homing coefficient. A–C, pDC-enriched leukocytes containing 4–7% pDC were obtained from C57BL/6 WT and CCR7-deficient mice bearing the Flt-3L–secreting tumor B16FL. Recipient mice received a total of $8 \times 10^7$ cells i.v. Shown are the homing coefficients for 120G8$^+$B220$^+$ pDC (A), 120G8$^-$B220$^+$ B cells (B), and 120G8$^-$B220$^-$ cells, which are primarily T cells (C; $n = 16$ mice analyzed in two independent experiments; dots indicate individual mice; bars indicate mean values). D, Same as A but pDC were derived from in vitro BM cultures using Flt-3L. Recipients received $8 \times 10^6$ BMpDC i.v. (dots represent individual mice; bars indicate mean values; data were derived from two independent experiments).

**FIGURE 6.** CCR7-dependent migration upon pDC activation and inflammation in vivo. C57BL/6 WT and CCR7-deficient BMpDC were harvested on day 8 of culture and stimulated with 2 $\mu$g/ml R848 overnight (no stimulation for C). WT and CCR7-deficient cells were then split and differentially labeled with either TAMRA or DDAO. DDAO-labeled WT cells were mixed with TAMRA-labeled CCR7-deficient cells at a 1:1 ratio. In cross-labeling experiments, TAMRA-labeled WT and DDAO-labeled CCR7-deficient cells were used. Mixtures of $8 \times 10^6$ pDC per mouse in total were i.v. injected into either untreated WT C57BL/6 recipient mice (A) or C57BL/6 WT recipient mice that were s.c. injected with cholera toxin in both flanks 1 d before the transfer (B). Two hours after transfer the mice were sacrificed and the homing of 120G8$^+$B220$^+$ Siglec-H$^+$CD11b$^+$ BMpDC was analyzed by flow cytometry. A, The ratio of transferred activated CCR7-deficient versus WT pDC was determined and shown as the homing coefficient. B–C, Results of two independent experiments are shown ($n = 13$; dots represent individual mice; bars indicate mean values). B, In cholera toxin-treated recipient mice the ratio of activated CCR7-deficient versus WT pDC was determined in SPL, draining (inguinal) LN (drLN), nondraining PLN (non drLN), and MLN. Results of two independent experiments are shown ($n = 10$; dots represent individual mice; bars indicate mean values). C, The ratio of transferred resting CCR7-deficient versus WT pDC was determined in SPL, draining (inguinal) LN (drLN), nondraining PLN (non drLN), and MLN. Results of two independent experiments are shown ($n = 11$; dots represent individual mice; bars indicate mean values).
well as in the MLN. Notably, we observed also lower pDC frequencies in blood, and in such case the reduced numbers of pDC in LN could result from a scarcity of supply. However, this is unlikely since pDC were found in regular amounts in BM, SPL, the IEL compartment, and liver. Moreover, CCR7 expression on pDC clearly affects entry into LN as shown by the adoptive cell transfers. In this study, a vast surplus of pDC is offered for entry into LN via the bloodstream, and yet the picture that emerged following transfer exactly matched that of the steady-state analysis in non-manipulated mice: a disequilibrium of transferred WT/CCR7 \(^{-/-}\) cells correlates with reduced pDC counts in steady-state in LN, whereas in SPL and BM regular pDC numbers go along with an unbiased immigration of exogenously provided cells. Although these observations identify hampered cell entry as a prime candidate for paucity of pDC in CCR7 \(^{-/-}\) LN, other factors might contribute to the strong reduction of pDC residing in the non-inflamed LN. Thus, it seems conceivable that pDC retention within the LN could be additionally affected in CCR7-deficient mice. In this scenario, a shortened retention period of pDC would not necessarily rely on direct interaction with CCR7. More likely, other cell types such as T cells as well as DC, which are also strongly reduced in CCR7-deficient mice, fail to provide factors that might help to retain pDC in LN following their homing. Similarly, missing contacts to either cells or diffusible mediators may shorten the half live of pDC in LN.

Given an intrinsic defect in the entry of pDC into the LN, CCR7 deficiency could be accompanied by profound changes in the expression of other homing molecules. To this end, we controlled the expression of CD11a, CD18, CD62L, and CXCRI3 but could not find differences between WT and CCR7-deficient cells as tested for pDC, CD4 T cells, and B cells. Thus, the most likely explanation for the defects observed in CCR7-deficient pDC is the lack of CCR7 itself. The current model of HEV extravasation established for T cells involves CD62L-dependent rolling, followed by LFA-1–mediated adhesion in which the integrin affinity is controlled by CCR7 signaling. Whereas naive T cells express high levels of CD62L, this selectin is expressed at rather low levels in pDC, which might help to explain why pDC less efficiently enter LN than do T cells. However, most resting pDC readily adhere to HEV under steady-state conditions (13), indicating that L-selectin levels expressed by pDC are sufficient to mediate firm adhesion to HEV. Of note, CD62L expression on pDC had been reported to be subjected to rapid modulation depending on the localization of these cells. pDC were shown to display high levels of CD62L in blood (21) but little or no CD62L in LN (7, 32). Analyzing pDC isolated from different organs, we were not able to confirm such differences. Instead, pDC display a quite consistent yet low expression of CD62L throughout SPL, blood, PLN, MLN, BM, and liver. Together with the high expression of CD11a/CD18, pDC share similarities with CD62L \(^{low}\) effector/memory rather than naive T cells regarding adhesion molecules (Supplemental Fig. 2B). This suggests an inverse correlation between naive and activated T cells and pDC, respectively. Naïve pDC tend to settle LN and their periphery just as Ag-experienced T cells would do. In contrast, activated pDC target their migration toward LN very much similar to how naive T cells would do during their recirculation.

Resting WT BMpDC displayed little but consistent migration toward CCL19 and CCL21. As expected, we observed a more robust migration toward these chemokines in activated pDC. The latter result is in line with previous reports demonstrating CCR7 induction and concomitant gain of migratory response to CCL19 and CCL21 upon pDC stimulation (16, 22). Therefore, in addition to the steady-state homing, we investigated the impact of CCR7 deficiency on the LN homing of activated pDC. R848-stimulated WT and CCR7-deficient pDC were transferred into either previously untreated mice or mice that were inoculated with cholera toxin s.c. the day before. Indeed, CCR7-deficient activated pDC were also impaired in entering LN compared with WT cells. Surprisingly, the differences in homing efficacy were comparable to those observed in resting pDC despite the profound differences in CCR7 expression levels between resting and activated WT pDC. This suggests that in both homeostatic trafficking and homing of activated pDC, other CCR7-independent mechanisms are also involved. Additionally, we tested the recruitment of resting pDC into inflamed LN. This setting probably fits best to physiologic migration of pDC into LN after peripheral injury or infection. As in all other combinations tested so far, even in this study, we find a similar reduction in the LN entry of CCR7-deficient pDC. These results point to an essential contribution of CCR7 in the LN homing of pDC regardless of their activation status of the pDC and also irrespective of the inflammatory milieu and concomitant alterations of chemoattractants found on the HEV.

Conversely, in plt mice, which lack CCR7 ligands on HEV, WT and CCR7-deficient pDC home to LN with equal efficiency, yet at very low numbers only (data not shown).

It has been published that upon pDC activation the chemokine receptor repertoire on these cells changes dramatically. While CCR7 is upregulated, CXCRI3, CXCR4, CCR5, and CCR9 are downregulated (14, 16, 28). This switch in receptor expression hints toward a more dominant role of CCR7 under these conditions. More importantly, the differential regulation of chemokine receptors on pDC upon stimulation most likely results in an overall altered migration pattern. Under steady-state conditions, pDC have the ability to enter both peripheral tissues and secondary lymphoid organs to acquire self Ags and to induce tolerance. Remarkably, pDC that acquired Ag in peripheral tissues were found in the blood but not the efferent lymph, suggesting a direct re-entering into the blood stream (12). Under inflammatory conditions, resting pDC may enter sites of infections, acquire Ag, and get stimulated. Increased CCR7 at the expense of CXCR3 and CCR9 may now allow an even more targeted migration toward lymphoid organs.

Additionally, the substantially increased levels of CCR7 upon pDC activation could affect their characteristics beyond the entry into the LN. It will have to be addressed in future studies how CCR7 expression and responsiveness affects LN internal homeostasis such as intranodal motility and migration velocity of the pDC. Other interesting aspects comprise the impact of CCR7 on pDC retention within the T zone, on LN exiting mechanisms as well as DC relevant functions such as polarization and endocytotic capacity. The concerted functions of CCR7 on pDC could thereby define CCR7 as a master regulator for this cell type, giving new insights into the complex phenotype of CCR7 deficiency, especially in terms of the impaired tolerance induction as shown for CCR7-deficient mice in models for oral and respiratory tolerance (4, 6). Because pDC have been identified to also essentially contribute to tolerance induction, including cardiac allografts (12), it will be interesting to address the role of CCR7 on pDC in models of allogeneic organ transplantation.

In conclusion, we show in this study that CCR7 expressed on resting murine pDC contributes substantially to pDC LN homing, a finding that is in line with compromised tolerance induction in CCR7-deficient mice. Additionally, CCR7 also affects the homing of activated pDC into steady-state and inflamed LN, rendering this chemokine critically involved in pDC migration regardless of their activation status.

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