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CCR7 Plays No Appreciable Role in Trafficking of Central Memory CD4 T Cells to Lymph Nodes

Bryan Vander Lugt,*‡,§ Noah J. Tubo,*‡,§ Suzanne T. Nizza,*‡,§ Marianne Boes,* Bernard Malissen,† Robert C. Fuhlbrigge,*‡,§ Thomas S. Kupper,*‡,§ and James J. Campbell*‡,§

CCR7−/− mice exhibit profound anomalies in lymph node and spleen architecture, which complicates the study of CCR7-mediated T cell trafficking in vivo. To circumvent this problem, we established in vivo models in which wild-type and CCR7−/− populations coexist within mice possessing normal lymphoid organs and must compete for developmental niches within the tissues of these mice. Under the conditions we have created in vivo, we find the entry of memory CD4 T cells into lymph nodes from the blood to be independent of CCR7. Thus, the central memory CD4 T cells that traffic though lymph nodes, which are often defined by their expression of CCR7, do not appear to gain any competitive homing advantage by expressing this receptor. Furthermore, in contrast to cutaneous dendritic cell populations, we found that CCR7 deficiency had no appreciable effect on the exit of CD4 T cells from inflamed skin. Finally, we found that wild-type and CCR7−/− precursors were equally represented within the major thymic subpopulations, despite previous findings that CCR7 plays a role in seeding the thymus from bone marrow–derived T cell precursors. The Journal of Immunology, 2013, 191: 3119–3127.

The leukocyte trafficking required for several basic immune functions, including the correct positioning of lymphocytes and dendritic cells (DC) during immune cell development, routine immunosurveillance by lymphocytes, and the generation of cognate immune responses. Proposed CCR7-mediated cell movements include the entry of bone marrow (BM)–derived T cell precursors into thymus (1–3), entry of naive (4–6) and central memory (7, 8) T cells into lymph nodes (LN) from blood through high endothelial venules (HEV), positioning of T cells within T cell zones of secondary lymphoid organs (SLO) (6, 9, 10), and the exit of T cells (11, 12) and mature DC (9, 13) from nonlymphoid tissues.

CCR7 is expressed by all naive lymphocyte subsets (14, 15), by central memory T cells (7, 8), and by mature migratory DC (13). Knockout (KO) mice lacking CCR7 or its ligands (CCL19 and CCL21) display numerous dramatic phenotypes, including pautocities in several T cell subpopulations and severe diminution in T cell–mediated Ag responses (10). The SLO of these mice are small, lack distinct T and B zones, and possess other histologically distinct irregularities (10). These diverse phenotypes have been construed as direct consequences of defective T cell trafficking (10, 16), but the potential indirect effects of T cell development within abnormal SLO microenvironments have not been fully explored.

We therefore created models in which CCR7-deficient T cell precursors could develop within relatively normal in vivo environments. In one such model, we reconstituted lethally irradiated hosts with mixed wild-type (WT) and CCR7-deficient BM. The presence of WT BM-derived cells preserved normal SLO architecture within recipients. This experimental design allowed us to directly compare trafficking behaviors between WT- and CCR7−/−-derived T cell subpopulations within each chimera. We have found that many T cell phenotypes associated with CCR7 deficiency are most likely secondary effects of development within abnormal SLO microenvironments. Contrary to previous thought, our findings do not support the notion that CCR7 plays a discernable role in the trafficking of Ag-experienced CD4 T cells to the LN, either directly from the blood or from peripheral tissues such a skin.

Materials and Methods

Mice

All experiments were performed with mice on the C57BL/6 background. Langerin-EGFP (LangEGFP) mice were provided by B. Malissen. CCR7−/− and leukotriene α (LTα)−/− mice were obtained from The Jackson Laboratory. C57BL/6, congenic CD45.1, and OT-II mice were obtained from Charles River Laboratories. Animal housing and experimentation was in accordance with institutional guidelines.

Flow cytometry analysis and sorting

Directed conjugated Abs were purchased from eBioscience and BioLegend. E-selectin–Fc chimera was purchased from R&D Systems, and anti-human Fcγ was purchased from The Jackson Laboratory. Single-cell suspensions were stained on ice and analyzed on a BD FACSCalibur six-color flow cytometer using FACSDiva software (BD Biosciences). Data analysis was done using FlowJo software (Tree Star). Naive OT-II T cells were sorted using a core facility LSRII (BD Biosciences).

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Abbreviations used in this article: BM, bone marrow; BMC, bone marrow chimeric; DC, dendritic cell; DNFβ, 2,4-dinitro-1-fluorobenzene; DP, double-positive; E-lig, ligand for E-selectin; HEV, high endothelial venule; KO, knockout; LangEGFP, Langerin-EGFP, LC, Langerhans cell; LN, lymph node; LTα, leukotriene α; MHC II, MHC class II; sdLN, skin-draining lymph node; SLO, secondary lymphoid organ; SP, single-positive; WT, wild-type.

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BM chimera generation and analysis
CCR7-competitive BM chimera (BMC)-F1 CD45.1/CD45.2 mice were irradiated with two doses of 600 rad separated by 3 h. Mice were immediately reconstituted with 5 × 10^6 RBC-depleted BM cells comprised of 1:1 WT(CD45.1)/CCR7^-/- (CD45.2) BM. Twelve weeks after reconstitution, mice were used for experiments as indicated. WT and KO donor populations were distinguished by congenic markers, and ratios were calculated using absolute numbers. Langerhans cell (LC) BMC-CCR7^-/- LangEGFP or CCR7^-/- LangEGFP mice were irradiated and reconstituted with WT BM as above. Ears were treated to remove hair (commercial Nair; Church & Dwight), split into dorsal and ventral halves, and floated on 1 mg/ml Dispase II (Roche) in PBS for 30 min to separate epidermis from dermis. Epidermal sheets were directly analyzed by epifluorescent microscopy.

Short-term homing assays
For blood-homing assays, 5 × 10^7 LN and splenic lymphocytes from CD45.1 CCR7^-/- and CD45.2 CCR7^-/- mixed 1:1 were injected retro-orbitally into recipient CD45.1/CD45.2 F1 mice. Two or 8 h after transfer, spleen and skin-draining LN (sdLN) were collected and analyzed by flow cytometry. For footpad homing assays, 5 × 10^7 mixed splenocytes were injected into the footpad of recipient mice. Popliteal LN were collected 18 h after transfer for analysis by flow cytometry.

2,4-Dinitro-1-fluorobenzene contact hypersensitivity response
A total of 50 μl 0.5% 2,4-dinitro-1-fluorobenzene (DNFB) in 4% aceton/ oil was painted onto shaved abdomen skin. Seven days after sensitization, mice were challenged with 5 μl 0.5% DNFB solution applied directly to ear skin. One day after challenge, mice were treated with 25 μg FTY720 (Cayman Chemical) i.p. Ears and sdLN were collected 2 d after FTY720 treatment.

Isolation of skin-infiltrating T cells
Ears were separated into dorsal and ventral halves and finely minced. Minced tissue was placed into 20 ml isolation medium (HBSS supplemented with 10 mM HEPES and 5 mM EDTA) at 4˚C with agitation by stir bar for 4–6 h. Supernatant containing released lymphocytes was then passed through a 40-μm filter and directly analyzed by flow cytometry.

Ag-specific responses
For immunization, mice were immunized epicutaneously as previously described (17). Briefly, Scotch tape (3M) was used to gently remove the cornified layer of ear skin, and then skin was treated with acetone and cholera toxin adjuvant before administration of chicken OVA23,339 peptide. For most OT-II experiments, 5 × 10^6 OT-II splenocytes were transferred retro-orbitally into recipient mice 24 h prior to immunization. For memory OT-II experiments, 500 purified naive OT-II T cells were transferred.

Results
Generating competitive BMC
We created competitive WT/CCR7^-/- mixed BMC (CCR7-BMC) similar to those we used previously to study CCR4 and CCR9 function in vivo (18–20). We reconstituted lethally irradiated WT hosts with 1:1 mixtures of BM from WT and CCR7^-/- donors. We used congenic CD45 variants to distinguish host (CD45.1/CD45.2 double-positive [DP]) from WT (CD45.1) and CCR7^-/- (CD45.2) donors. (Note: all DC subsets required for presenting Ag to T cells are available in these chimeras from the host and WT BM donor, despite the additional presence of CCR7^-/- DC populations.)

After ≥12 wk, we evaluated the relative contribution of each BM donor to individual cell populations within each host. A 1:1 ratio of WT-to-CCR7^-/- cells (i.e., equivalent to the input population) would indicate that CCR7 expression provided no competitive advantage for WT over CCR7^-/- cells. An n:1 ratio would indicate that CCR7 expression provided an n-fold advantage for WT over CCR7^-/- cells. To correct for random differences in BM engraftment among recipients, we normalized the ratio of each cell type to a reference population not influenced by CCR7 function for each recipient. We used peripheral blood monocytes and neutrophils for this purpose. These two populations did not differ significantly from each other in WT:CCR7^-/- ratio for any mouse used in these studies. Based on these two circulating leukocyte populations, the actual reconstitution efficiency ranged between 0.50 and 1.53 among individual recipients, in contrast to the ideal value of one (18–20).

Identifying naive and Ag-experienced T cell subsets
We used CD44 with either CD45RB (for CD4 T cells) or CD122 (for CD8 T cells) to distinguish between naive and Ag-experienced populations (note: in both cases, CD44lo defines naive cells, but CD45RB and CD122 help to establish the cutoff point between CD44hi and CD44lo cells). An example of our phenotypic characterization is shown in Fig. 1A, left panel. We confirmed that Ag-experienced CD4 and CD8 T cells defined by these criteria were overrepresented in the CCR7^-/- SLO, supporting our confidence in these immunophenotyping criteria (Supplemental Fig. 1) (10).

The presence of CCR7 on Ag-experienced T cells is a characteristic commonly used to identify the central memory subset, but this is not possible in a study involving CCR7^-/- mice. Instead, for the purposes of this study, we used an operational definition for central memory T cells (i.e., their ability to enter LN directly from the blood via HEV) (7, 8).

T cells that enter nonlymphoid tissues are often classified as effector memory (7, 8). However, many T cells that express markers required for entering nonlymphoid tissues (i.e., cutaneous lymphocyte Ag and α4β7-integrin) also express CCR7, the purported central memory marker (15). Thus, for the purposes of this study, we consider T cells found within nonlymphoid tissues to be enriched but not pure effector memory populations.

CCR7 does not influence accumulation of Ag-experienced T cells in LN
We found WT naive cells from sdLN to hold an ~20-fold competitive advantage over CCR7^-/- naive cells (Fig. 1A, 1B). In contrast, WT Ag-experienced T cells had no appreciable advantage over their CCR7^-/- counterparts. Data shown are from the cervical LN, but results were very similar for mesenteric LN (Supplemental Fig. 2). In the spleen, we observed a small but significant skewing in favor of the WT donor in naive but not Ag-experienced populations (Fig. 1B).

Similar trends were seen whether the tissue drainage area was inflamed (Fig. 1B, right panel) or resting (Fig. 1B, left panel). However, there was a very small but significant advantage for CCR7 expression by Ag-experienced CD4 but not CD8 populations within the inflamed LN.

CCR7 and thymic seeding
Several studies suggest that CCR7 plays a role in T cell development through its involvement in seeding the thymus with BM-derived T cell precursors (21, 22). However, our competitive assays reveal that thymic development proceeds with normal kinetics in the absence of CCR7. WT and CCR7^-/- donor-derived cells were equally represented within the immature CD4/CD8 DP population, as well as each of the single-positive (SP) populations (Fig. 1C).

Differential requirements for entry of CD4 versus CD8 central memory T cells into LN from the blood
Naive T cells are thought to possess only a single route through which they may enter LN: from blood via HEV (23) [not including intranodal migration after entry to a connected LN through HEV (9)]. However, Ag-experienced T cells can enter through the HEV or from peripheral tissues via the afferent lymph (24). As discussed above, those that enter through peripheral tissues are enriched in
FIGURE 1. Influence of CCR7 deficiency on accumulation of naive and Ag-experienced T cell subsets within lymphoid organs. Lethally irradiated mice (CD45.1/CD45.2) were reconstituted with a 1:1 mixture of BM from WT (CD45.1) and CCR7−/− (CD45.2) mice. Twelve weeks after reconstitution, naive and Ag-experienced (Ag Exp) CD4 and CD8 T cell populations from sdLN, spleen, and thymus were analyzed for the relative contribution of cells from each donor. (A) Plots show gating criteria for each population examined (left panel) and the representation of each cell from each donor within the indicated population (right panel). Data are shown from a single representative CCR7-BMC mouse. CD45.1/CD45.2 DP cells excluded from the gates in the right panel represent host-derived cells that survived irradiation. (B) WT/KO donor ratios of populations gated as in (A). Ratios were normalized to correct for engraftment efficiency of each donor BM type for each CCR7-BMC mouse as described in text. Left panel, Data analysis of six resting mice with each data point representing an individual mouse. Bars show mean ± SD. Similar data were obtained in at least six independent analyses of four to seven mice per analysis. Right panel, Data analysis of mice 3 d after cutaneous ear challenge of sensitized mice with DNFB. Each data point represents a single mouse from a three-mouse experiment. Similar data were obtained in three independent analyses of three to five mice per experiment. Calculation of p values was performed with a one-sample two-tailed t test against a hypothetical value of 1. (C) Thymocyte populations from competitive CCR7-BMCs. Left panel shows gating criteria for each population analyzed. Right panel shows data from thymocyte populations analyzed as in (B). Each data point represents a single mouse from a four-mouse experiment, representative of two experiments. n.s., No significant advantage detected for WT cells over CCR7−/− cells.
CCR7 IS DISPENSABLE FOR Ag-EXPERIENCED T CELL HOMING

Interestingly, CCR7 did convey a significant advantage to Ag-experienced CD8 T cells in the LN but not spleen. Thus, unlike CD4 T cells, central memory CD8 T cells (or at least a subset of them) do indeed appear to use CCR7 for entry into LN from blood. Differential representation of central versus effector memory T cells

In our short-term homing assay above, we further observed that the Ag-experienced-to-naive ratios within the recovered CD4 populations from both LN and spleen were greatly reduced with respect to those of the input population at the 8 h time point (Fig. 3, right panel). This was not the case for the CD8 populations, in which the ratios within the input and recovered populations were not significantly different (Fig. 3, right panel). [Please note: data from CCR7−/− donor-derived cells were not considered in Fig. 3].

Thus, a given Ag-experienced CD8 T cell in the circulation is most likely to enter a lymphoid tissue (just as likely as a naive CD8 T cell), but a given Ag-experienced CD4 T cell is more likely to enter a nonlymphoid tissue. The Ag-experienced CD8 T cell population within blood and lymphoid organs is therefore comprised primarily of central memory cells. In contrast, Ag-experienced CD4 T cells in the LN at any given time most likely migrated from nonlymphoid tissue and thus meet the operational definition of effector memory (7, 8).

WT and CCR7−/− T cells accumulate equally well within inflamed skin

Two recent studies propose that CCR7 is required for migration of T cells from peripheral tissues to the draining LN (11, 12). This is difficult to reconcile with our finding that Ag-experienced WT and CCR7−/− cells are equally represented within LN of our competitive chimeras (Fig. 2). If CCR7 were indeed necessary for emigration from peripheral tissue, one would expect WT Ag-experienced CD4 T cells to greatly outnumber their CCR7−/− counterparts, because the majority of Ag-experienced CD4 T cells within LN are likely to have arrived there from peripheral tissue (Fig. 3). Thus, our findings suggest that CCR7 is either unnecessary for this migration step or that some other mechanism compensates for the proposed deficiency.

Nonetheless, if CCR7-deficient CD4 T cells were able to enter peripheral tissues but unable to exit efficiently, one would expect CCR7−/− cells to accumulate disproportionately within the peripheral tissue. To test this notion, we used skin as a representative peripheral tissue and returned to the CCR7-competitive BMC model. We directed Ag-experienced T cells into the ear skin by inducing an anamnestic response through repeated topical immunization with OVA plus adjuvant (18). Interestingly, we found that WT and CCR7−/− cells accumulated equally within inflamed skin (Fig. 4A). Thus, in the case of skin, Ag-experienced WT and CCR7−/− T cells do not appear to use CCR7.

FIGURE 2. Central memory CD4 and CD8 T cells differ in their CCR7 requirements for homing to LN directly from blood. A 1:1 mixture of WT (CD45.1) and CCR7−/− (CD45.2) splenocytes was transferred i.v. into WT (CD45.1/CD45.2) recipient hosts. Two or eight hours after transfer, the sdLN and spleen of recipient mice were analyzed for the presence of donor cells. (A) Left panel shows the WT and KO components of the naive CD4 T cell compartment within the mixed donor input population prior to transfer. Right panel shows the WT and KO components of the naive CD4 T cell compartments recovered from sdLN and spleen of recipient mice after transfer. Data in these panels are from a single representative recipient mouse. CD45.1/CD45.2 DP cells excluded from the gates in the FACS plots are comprised entirely of host cells. (B) WT/KO ratios (normalized to that of the input population) calculated for donor T cell populations isolated 2 h (left panel) or 8 h (right panel) after transfer. Two-hour data are derived from two separate experiments of three recipient mice. Each eight-hour data are derived from eight recipient mice from a single experiment. Each data point represents an individual recipient mouse. Bars show mean ± SD. Calculation of p values was performed using a one-sample two-tailed t test against a hypothetical value of 1. n.s., No significant advantage detected for WT cells over CCR7−/− cells. AgExp, Ag-experienced.

FIGURE 3. Ag-experienced CD4 and CD8 T cell populations home differentially from blood to peripheral tissues versus lymphoid organs. The proportion of T cells within the WT CD4 and CD8 populations that displayed the Ag-experienced immunophenotype (as described in text) was calculated for input and recovered cell populations from the experiments reported in Fig. 2. Bar graphs show mean ± SD. Calculation of p values was performed using the Mann–Whitney U test. n.s., No significant difference from the input population.

effect cells, whereas those that enter through HEV are operationally defined as central memory cells (7, 8). The CCR7−/−BMC experiments shown in Fig. 1 provide a snapshot of T cell trafficking that represents the combined contributions of both processes.

We wished to directly examine HEV-specific homing from blood to LN and therefore designed a shorter-term model in which homing from the blood would reach near completion, but the vast majority of cells entering nonlymphoid tissues would not have sufficient time to enter the LN (25, 26). In this short-term competitive assay, we transferred 1:1 mixtures of splenocytes from mature WT and CCR7−/− donors into nonirradiated recipients and distinguished host-derived from donor-derived populations using the CD45 congenic markers described in Fig. 1. Lymphoid organs from the recipients were harvested for flow cytometry either 2 or 8 h after transfer. The ratio of WT to CCR7−/− cells within donor-derived populations was calculated and normalized to the input population (Fig. 2).

CCR7 conferred a marked competitive advantage to both CD4 and CD8 naive T cells for access to LN but not spleen (Fig. 2). Also, as seen in the BMC, WT and CCR7−/− Ag-experienced CD4 T cells were represented equally within LN and spleen. Thus, the central memory CD4 T cells that traffic through LN, which are often defined by their expression of CCR7 (7, 8), do not appear to gain any competitive homing advantage by expressing this receptor.
CCR7 and migration of DC to LN from peripheral tissues

As our findings regarding CCR7 and the exit of CD4 T cells from skin did not agree with the conclusions of previous studies, we next used the CCR7-BMC model to observe the accumulation of skin-derived DC within sdLN, for which CCR7 function has also been proposed as a requirement for exit from skin. Our model confirms that CCR7 expression confers a strong competitive advantage for accumulation of MHC class II (MHC II<sup>hi</sup>) migratory DC in sdLN (Fig. 5A). CCR7 also confers a small but significant advantage to MHC II<sup>lo</sup> populations within LN, especially when compared with the same populations from spleen (where CCR7 had no effect).

It was not possible to assess migration of LC to sdLN using the model in Fig. 5A, because LC precursors in skin are highly radiosensitive (29); the LC population in the CCR7-BMC model is comprised solely of host-derived cells, which are systematically excluded from analysis (30). We therefore modified our BM adoptive-transfer approach to directly assess the importance of CCR7 in LC migration.

We used the LangEGFP mouse strain that allows observation of LC by virtue of EGFP expression under the Langerin promoter (29). We bred this strain with the CCR7<sup>−/−</sup> strain to obtain CCR7<sup>+/−</sup>/EGFP<sup>+</sup> or CCR7<sup>−/−</sup>/EGFP<sup>+</sup> littermates, each serving as hosts for WT BM after lethal irradiation. Through this design, all EGFP<sup>+</sup> DC in the epidermis and sdLN are LC of host origin and were thus CCR7<sup>+/−</sup> or CCR7<sup>−/−</sup> depending on the host. All radiosensitive DC were of donor (WT) origin. Eight weeks after reconstitution, we found comparable densities of EGFP<sup>+</sup> cells within the epidermis of both chimera types (Fig. 5B, left panel). However, only CCR7<sup>−/−</sup> LC accumulated within the sdLN (Fig. 5B, right panel).

Tissue-selective homing capabilities of CCR7<sup>−/−</sup> T cells

The entry of CD4 T lymphocytes into skin is thought to require an imprinting step, whereby naive cells acquire skin-selective homing molecules after recognizing skin-derived Ag within the sdLN (24, 27, 31). As CCR7<sup>−/−</sup> naive T cells have great difficulty entering LN (Figs. 1, 2), one would expect them to have more restricted access to the imprinting apparatus than WT naive cells. It is therefore noteworthy that we found any CCR7<sup>−/−</sup> cells within skin at all (Fig. 4).

One of the most important molecules directly involved in skin-selective T cell homing is the carbohydrate ligand for E-selectin (E-lig; known as cutaneous lymphocyte Ag in humans) (32–34). CD103, the ligand of E-cadherin, is considered important for extended retention of T cells in skin (35).

We isolated CD4 T cells from the sdLN draining the inflamed ear skin of our CCR7-BMC mice to determine E-lig and CD103 expression (Fig. 6A). We found no significant difference between the WT and CCR7<sup>−/−</sup> populations regarding expression of either molecule. Furthermore, when gating specifically on the CD4<sup>+</sup>/E-lig<sup>+</sup> population from CCR7-BMC mice, we observed a 1:1 ratio for WT and CCR7<sup>−/−</sup> cells (Fig. 6B).

Imprinting of Ag-specific T cells with E-lig during the primary immune response

The data shown in Fig. 6A derive from mice immunized multiple times with OVA plus adjuvant prior to analysis. As we have shown Ag-experienced CCR7<sup>−/−</sup> T cells to have normal access to skin (Fig. 4), it is possible that the observed skin-specific imprinting of time period when new T cells are prevented from entering. However, the CD45.1/CD45.2 ratio remained ~1:1 under all conditions tested (Fig. 4B). If CCR7 were required for T cell emigration from skin, one would have expected CCR7<sup>−/−</sup> cells to remain trapped in the ear skin, whereas the WT cells exited efficiently, resulting in a decreased WT/CCR7<sup>−/−</sup> ratio, which was not the case.

Efficient exit of CCR7<sup>−/−</sup> CD4 T cells from skin

To further evaluate the role of CCR7 in T cell emigration from peripheral tissues, we sought to isolate the tissue-emigration step from the other elements contributing to accumulation within LN. [Note: this experiment focuses specifically on Ag-experienced cells because naive cells do not home to nonlymphoid tissues (24, 27).] Using our CCR7-BMC model, we allowed T cells to accumulate within inflamed ear skin for 2 d, at which time we inhibited further infiltration into the skin. The third group was not further manipulated until day 4, when both the second and third groups were harvested for T cell isolation from skin. Each dot represents the CD45.1/CD45.2 ratio for CD4<sup>+</sup> CD4<sup>+</sup> cells isolated from both ears of a single CCR7-BMC mouse. Calculation of p values was performed using an unpaired two-tailed t test, n.s., No significant difference was detected between the WT and CCR7<sup>−/−</sup> populations.
CCR7−/− T cells occurred in the Ag-experienced population rather than conventionally in the naive population.

We therefore wished to directly observe the relative efficiency with which WT versus CCR7−/− cells became imprinted during a primary immune response. It was not feasible to track Ag-responsive endogenous naive T cells after a primary immune response due to the expected small numbers of responding cells. We instead used a TCR-transgenic adoptive-transfer model. CD4 splenocytes from OT-II mice (which express a transgenic TCR for OVA) were transferred into host mice (17, 19). The OT-II donors were bred onto either the WT or the CCR7−/− background.

Tissue-specific imprinting is believed to occur within the nodes that immediately drain the site of inflammation (24, 27). If our experiments were to show that CCR7−/− naive OT-II cells could be imprinted normally, it would raise the possibility that sdLN are unnecessary for skin-selective imprinting. We therefore included LTα−/− recipients as a control: LTα−/− mice have no detectable peripheral LN and thus provide a baseline for skin-specific homing without an sdLN contribution.

We topically immunized the ears once with OVA plus adjuvant (17, 19). Skin-infiltrating cells were isolated from the inflamed skin 5 d later, and OT-II cells were enumerated (Fig. 7A). As shown previously in this model, accumulation of WT OT-II cells within normal recipients was relatively large [note: only negligible numbers of OT-II cells are found within skin inflamed with adjuvant alone (17, 19)]. Strikingly, very few CCR7−/− cells accumulated within normal ear skin, and very few WT OT-II cells accumulated within LTα−/− ear skin. The functional spleen possessed by LTα−/− mice is therefore not sufficient to replace sdLN for imprinting naive T cells.

In similar experiments, we examined OT-II cells within the sdLN of recipient mice (Fig. 7B). We found CCR7−/− OT-II cells to be severely impaired in their ability to become imprinted for skin-selective homing. This was most likely due to the paucity of CCR7−/− naive cells within the sdLN, implying dramatically lower numbers of CCR7−/− OT-II cells available to undergo the imprinting process. (Note: it is not possible to present sdLN data from LTα−/− mice, as they do not possess any LN.)

Discussion
We have for the first time, to our knowledge, assessed the contribution of CCR7 to various T lymphocyte and DC homing processes in vivo without the confounding variable of T cell development within grossly aberrant lymphoid organs. We find that CCR7 is not involved in the migration of central memory CD4 T cells to LN through the HEV, nor is it involved in the exit of lymphocytes from peripheral tissue (i.e., skin).
Thymic development

Our CCR7-BMC system does not show that developing thymocytes gain any advantage by expressing CCR7. Both WT and CCR7$^{-/-}$ thymocytes were equally represented in the DP and SP populations (Fig. 1C). Although other evidence supports a role for CCR7 (along with CCR9) in seeding the thymus with BM-derived T cell precursors (1, 2, 36, 37), this role appears to be diluted in later developmental stages, perhaps due to prodigious expansion during the DP stage (38). However, the effect of CCR7 deficiency is dramatically different from that of CCR9 deficiency, in which CCR9$^{-/-}$ cells were 10-fold less abundant than WT cells at most stages of thymic development (20, 39).

A second role proposed for CCR7 in thymus is the migration of positively selected SP cells from the cortex to the medulla (40, 41). Our findings in the CCR7-BMC are not inconsistent with such a role: CCR7 had a small but significant effect on naive CD4 and CD8 numbers within the spleen. As homing from blood to spleen does not involve HEV [and thus is not thought to require CCR7 (24, 42)], it is possible that this finding reflects a slightly lower output of CCR7$^{-/-}$ naive T cells from the thymus.

Homing of naive T cells to LN from blood

Naive WT cells had a very strong advantage over naive CCR7$^{-/-}$ cells in both the CD4 and CD8 LN populations, in some cases reflecting a $>20$-fold difference (Figs. 1, 2). Regardless of the reason for slightly fewer CCR7$^{+}$ naive T cells in the spleen, the dramatically larger influence of CCR7 on accumulation of naive T cells in LN compared with spleen confirms the crucial role of this receptor in homing of naive T cells to from the blood to LN via the HEV.

Homing of central memory cells to LN from blood

For the purposes of this study, we operationally defined central memory T cells as those capable of entering the LN directly from blood. Although expression of CCR7 was the original marker proposed for central memory T cells (7, 8), we find this receptor to have no demonstrable role in the entry of central memory CD4 T cells into LN from the blood (Fig. 2). Our assays require direct competition between WT and CCR7$^{-/-}$ populations for accumulation within specific niches of a given tissue or organ and thus would allow detection of even a redundant role for CCR7, but this was not seen. Although it has been previously reported that some Ag-experienced T subsets possess alternative, less efficient CCR7-independent mechanisms for homing to LN (43–45), it was unexpected that CCR7 would convey no detectable competitive advantage whatsoever. Thus, it appears that CCR7 plays no role in the defining function of central memory T cells.

CD8 central memory T cells, in contrast, were characterized by a significant CCR7 requirement for LN entry from blood (Fig. 2). This dependence was somewhat less than that of naive CD8 T cells, consistent with the notion that CD8 central memory T cells do indeed possess alternative (but less efficient) CCR7-independent mechanisms for LN entry from blood (43–45). It was also noteworthy that Ag-experienced CD8 T cells were more likely to enter LN from the blood than Ag-experienced CD4 T cells (Fig. 3). This suggests that the circulating Ag-experienced CD8 T cell population is comprised mostly of central memory cells, whereas...
CCR7 is dispensable for Ag-experienced T cell homing

Our finding that WT and CCR7\(^{-/-}\) CD4 T cells in our CCR7-BMC transgenic recipients (which lack peripheral LN but possess a functional spleen) were found within the inflamed skin when normal OT-II cells were detectable within the inflamed skin when normal OT-II cells were transferred into LTo\(^{-/-}\) recipients (which lack peripheral LN but possess a functional spleen). Thus, both peripheral LN and functional CCR7 are required to develop a skin-specific primary immune response.

This requirement for CCR7 in skin-selective imprinting during primary but not recall immune responses raises the possibility of an alternative tissue-selective imprinting mechanism, which does not require the presence of naive T cells in the sdLN. One possible mechanism would involve the CCR7\(^{-/-}\) Ag-experienced T cells present in the sdLN of our CCR7-BMC during the resting state.
Perhaps such Ag-experienced cells have the ability to gain skin-selective homing capabilities within this environment, much like normal naive cells.

In conclusion, we find that multiple effects of CCR7 deficiency previously attributed to the functional role of CCR7 in T lymphocyte trafficking can be traced back to secondary effects from T cell development within the abnormal LN and splenic environments characteristic of mice lacking CCR7 or its ligands. We were able to uncover these new insights by comparing the behavior of WT versus CCR7−/− cells within in which the normal lymphoid environment had been reconstituted.

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Disclosures

The authors have no financial conflicts of interest.

References

Figure S1. Antigen-experienced T cells are disproportionately represented in CCR7<sup>-/-</sup> lymphoid tissue. sdLNs and spleen were collected from WT and CCR7<sup>-/-</sup> mice and analyzed by flow cytometry. Proportions of naïve (CD4: CD44<sub>lo</sub>/CD45RB<sub>hi</sub>, CD8:CD44<sub>lo</sub>/CD122<sub>lo</sub>) and antigen-experienced (CD4: CD44<sub>hi</sub>/CD45RB<sub>lo</sub>, CD8: CD44<sub>hi</sub>/CD122<sub>hi</sub>) were determined for gated CD4 and CD8 populations.
Figure S2. Antigen-experienced T cell long-term accumulation in gut-draining LNs is also CCR7-independent. (a) MLNs were collected from CCR7 competitive BMCs and analyzed as in Fig 1a&b. Normalized WT:KO donor ratios are graphed with each data point representing a single mouse from a 5 mouse experiment representative of 3 experiments. (b) Inflammatory T cell homing was determined from MLNs collected from mice after 7 days of treatment with 5% DSS in drinking water.
Figure S3. Adoptive Transfer Into Footpad May Indicate Advantage for CCR7 for Homing from Peripheral Tissue. Splenocytes from CD45.1 WT and CD45.2 CCR7-/ donors were mixed 1:1. Mixed splenocytes (5x10^7) were injected into the footpads of CD45.1/CD45.2 F1 mice. Eighteen hours after injection, popliteal LNs were collected to determine donor contribution. Spleen was simultaneously analyzed as a control for contamination of blood-homing transferred cells. Graph displays cumulative data from 5 experiments as mean+/- SEM.