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*J Immunol* published online 23 October 2015
http://www.jimmunol.org/content/early/2015/10/23/jimmunol.1500993

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2015/10/23/jimmunol.1500993.DCSupplemental

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CD8 T Cells Enter the Splenic T Cell Zones Independently of CCR7, but the Subsequent Expansion and Trafficking Patterns of Effector T Cells after Infection Are Dysregulated in the Absence of CCR7 Migratory Cues

Naveen Sharma,*-1 Alexandre P. Benechet,* Leo Lefrançois,*-2 and Kamal M. Khanna*,#

CCR7 is an important chemokine receptor that regulates T cell trafficking and compartmentalization within secondary lymphoid organs. However, the T cell–intrinsic role of CCR7 during infection in the spleen is not well understood. This study was designed to understand how CCR7-dependent localization and migration of CD8+ T cells in different compartments of the spleen affected the primary and recall responses after infection. To this end, we used adoptive transfer of naive Ag-specific CD8 T cells (OT-I) that either lacked CCR7 or constitutively expressed CCR7 (CD2-CCR7) in mice that were subsequently infected i.v. with *Listeria monocytogenes*. We show that naive CCR7+/−/CD8+ T cells failed to enter the T cell zone, whereas CD2-CCR7 OT-I cells were exclusively confined to the T cell zones of the spleen. Surprisingly, however, CCR7−/− OT-I cells entered the T cell zones after infection, but the entry and egress migratory pattern of these cells was dysregulated and very distinct compared with wild-type OT-I cells. Moreover, CCR7-deficient OT-I cells failed to expand robustly when compared with wild-type OT-I cells and were preferentially skewed toward a short-lived effector cell differentiation pattern. Interestingly, CCR7−/−, CD2-CCR7, and wild-type OT-I memory cells responded equally well to rechallenge infection. These results highlight a novel role of CCR7 in regulating effector CD8 T cell migration in the spleen and demonstrate differential requirement of CCR7 for primary and secondary CD8 T cell responses to infection. The Journal of Immunology, 2015, 195: 000–000.

The spleen is the most important priming site for generating CD8 T cell immunity against blood-borne pathogens such as *Listeria monocytogenes*. The anatomical structure of the spleen is compartmentalized into white pulp (WP), which is a T cell–rich area surrounded by B cell follicles, and the red pulp (RP), which is a blood-filled area and contains populations of macrophages, dendritic cells (DCs), and granulocytes (1). The WP and RP are separated by the marginal zone (MZ), where specific subsets of macrophages as well as CD21high B cells reside. The uptake of *L. monocytogenes* by CD8α+ DCs and their entry into the WP is shown to be an important step in the initiation of the CD8 T cell immune response against *L. monocytogenes* (2, 3). CD8α+ DCs capture and transport the bacteria to the splenic WP where CD8 T cells encounter *L. monocytogenes*–derived Ags. A robust CD8+ T cell response is required for protective immunity against intracellular pathogens such as *L. monocytogenes*.

Naive T cells recirculate between peripheral blood and secondary lymphoid organs in search of Ag (4). When naive CD8+ T cells encounter cognate Ag, they undergo an expansion phase giving rise to a large number of effector cells at the peak of response (5). Subsequently, most of these effector CD8 T cells die and only a small heterogeneous population of memory CD8+ T cells survives. These memory CD8+ T cells can be further divided into effector memory CD8+ T cells or central memory CD8+ T cells on the basis of CCR7 and CD62L surface protein expression. Central memory CD8+ T cells express CCR7 or CD62L and thus mainly reside in lymph nodes and the splenic T cell zones. Conversely, effector memory CD8+ T cells fail to express CCR7 or CD62L and consequently inhabit nonlymphoid organs and the splenic RP (6, 7). The generation of memory cells can be affected by many factors, including strength and duration of antigenic stimulus, inflammatory milieu, and modulation of chemokine and homing receptors (8–12).

Mounting a protective immune response is critically dependent on the orchestrated movement of cells within lymphoid organs. Secondary lymphoid organ structure is one of the underlying regulators of immune responses and is responsible for promoting interactions between cells as well as between cells and extracellular matrix. How local migratory properties of naive or memory CD8 T cells affect priming or recall responses, respectively, is not well understood. In this vein, the role of CCR7 is of particular interest given that CCR7 is a critical regulator of naive, effector, as well as memory CD8 T cell migration. CCR7 plays an important role in migration of T cells to and within secondary lymphoid organs. Naive CD8 T cells fail to migrate to lymph nodes and Peyer’s patches of CCR7-deficient mice. Although, CCR7−/−
CD8 T cells migrate to the spleen, they however fail to travel to the splenic T cell zones and remain in the RP (13–16). Conversely, constitutive expression of CCR7 (i.e., in CD2-CCR7 transgenic mice) restricts naive CD8 T cells exclusively to the splenic WP (16). In the present study we took advantage of the aforementioned unique properties of CCR7+/− and CD2-CCR7 transgenic mice to understand how the local compartmentalization of naive or memory CD8 T cells within the spleen impacts the priming and migration of naive, or reactivation of memory, CD8 T cells following primary or secondary infection, respectively. To this end, we investigated whether naive CD8 T cells, which are found in both the splenic T cell zones and in the RP, are capable of encountering Ag in the RP or WP, and whether the location of the initial priming site within the splenic tissue alters the magnitude or phenotype of effecter CD8 T cell expansion and differentiation. Additionally, because effector memory CD8 T cells fail to express CCR7 or CD62L, and thus remain in the RP, we wanted to determine whether these cells migrate to the WP to encounter Ag or whether they can encounter Ag within the RP after a secondary infection. As with naive CD8 T cells, we also investigated whether the location of the initial Ag re-exposure affects memory CD8 T cell recall response.

We addressed this question by comparing OVA-specific OT-I TCR transgenic CD8 T cells devoid of CCR7 (CCR7−/− OT-I), which restrict themselves to RP of the spleen, and CD2-CCR7 OT-I cells, which constitutively express CCR7 under CD2 promoter and therefore are exclusively found in the splenic T cell zones. Our results unexpectedly showed that in response to primary or secondary infection CCR7+/− naive or memory CD8 T cells in fact migrated to the splenic T cell and B cell zones. Although early after infection CCR7+/− effector T cells were capable of entering the T cell zones, the anatomical program followed by these cells after Ag presentation was very distinct from that of the wild-type (WT) effector CD8 T cells. CCR7−/− effector OT-I cells exited the T cell zones with accelerated kinetics and failed to use the bridging channels, apparently migrating through the B cell follicles into the RP (17). The efficiency of the primary CD8 T cell immune response was dependent on CCR7-mediated migratory cues, because CCR7+/− CD8 T cells failed to expand as well as WT CD8 T cells. Interestingly, in contrast to the naive CD8 T cell response, the memory CD8 T cell response to a secondary infection was not as dependent on CCR7-mediated migratory cues because CCR7+/− memory CD8 T cells expanded normally after rechallenge. Thus, our study revealed novel mechanisms by which CCR7 regulates effector CD8 T cell egress kinetics in the spleen and influences Ag-specific CD8 T cell responses to an infection.

Materials and Methods

Mice

C57BL/6J mice were purchased from Charles River Laboratories through the National Cancer Institute animal program. The OT-I mouse line was provided by Dr. W.R. Heath (Walter and Eliza Hall Institute, Parkville, VIC, Australia) and Dr. F. Carbone (Monash Medical School, Prahan, VIC, Australia) and was maintained as a C57BL/6-Cd45.1-RAG−/− line. The transgenic CD2-CCR7 OT-I mouse line was generated by crossing the C57BL/6-Cd45.1-RAG−/− line with the CD2-CCR7 transgenic line provided by Dr. Andrew D. Luster (Massachusetts General Hospital, Harvard University, Boston, MA).

Infections

Recombinant L. monocytogenes–producing OVA (LM-OVA) was produced as previously described (17). The actA− LM-OVA was a gift of Dr. John T. Harty (University of Iowa, Iowa City, IA). Unless otherwise mentioned, for all experiments mice were immunized i.v. with 10^5 CFU actA− L. monocytogenes.

Isolation of lymphocytes

Single-cell suspensions were prepared from spleens after infections at different time points, and lymphocytes were released from the tissue by digestion with 100 U/ml collagenase (In vitrogen, Carlsbad, CA) in RPMI 1640 containing 1 mM MgCl2, 1 mM CaCl2, and 10% FCS at 37°C for 30 min. RBCs were lysed with ammonium chloride, and lymphocytes were filtered through cell strainers.

Immunofluorescence analysis

At the indicated times after infection, lymphocytes were isolated and OT-I cells were detected using a CD45.1-specific mAb. For staining, lymphocytes were suspended in PBS/2% FCS/0.05% NaN3/0.05% NaN3 at a concentration of 1 × 10^6 to 1 × 10^7 cells/ml, followed by incubation at 4°C for 30 min with appropriate dilutions and combinations of Abs specific for CD8, CD127, KLRG1, CD69, CD62L, CD11a, and CD45.1 (Bio-Legend, San Diego, CA; BD Biosciences, San Jose, CA; or eBioscience, San Diego, CA). Relative fluorescence intensities of up to eight fluorochromes in a single stain were measured with an LSR II (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

CFSE labeling and detection

OT-I cells were harvested from spleens of different naive CD45.1+ OT-I mice. Cells were stained with 1 μM of CFDA-SE dye in PBS (Vybrant CFSE/SE dye from Life Technologies). Cells were incubated in a 37°C water bath for 12–15 min. Two to three times the volume of media containing serum was added. CFSE-labeled cells were spun down and suspended in PBS. CFSE-labeled cells (0.5 million) were transferred to naive congenic CD45.2− mice 1 d prior to infection with 10^5 LM-OVA (actA−). Spleens were harvested at different days and analyzed for CFSE and CD45.1 by flow cytometry using an LSR II.

Intracellular detection of cytokines

Lymphocytes were isolated from the indicated tissues and cultured for 5 h with 1 mg/ml BD GolgiStop (BD Biosciences) with or without 1 mg/ml of the OVA-derived peptide SIINFEKL. After culture, cells were stained for surface molecules and then fixed, and cell membranes were permeabilized in BD Cytofix/Cytoperm solution (BD Biosciences) and stained with anti–IFN-γ, anti–IL-2, or anti–TNF-α mAbs or the appropriate corresponding isotype control rat IgG (BD Biosciences). Cells were then washed, and the fluorescence intensity was measured on an LSR II.

Whole-mount confocal laser microscopy

Mice were sacrificed at the indicated times after infection, and the spleens were excised and processed for staining. Briefly, thick sections of whole spleen tissue were cut using a vibratome. Tissues were fixed in 2% paraformaldehyde for 2 h at 4°C. Tissues were subsequently washed and incubated overnight at 4°C in round-bottom 96-well plates with fluorescently labeled anti–CD45.1, B220, CD8 (Bio-Legend), biotin-CD169 (Serotec), anti-mouse CD11c (eBioscience), and biotin-CD11c (eBioscience) diluted in 2% normal goat serum and then incubated with rat anti–biotin Cy3 (Bio-Legend). After washing, tissues were mounted, and analyzed using an LSM 780 confocal microscope (Zeiss, Oberkochen, Germany). Image analysis was performed using Imaris software (Bitplane, St. Paul, MN).

Memory transfer experiments

Memory OT-I CD8 T cells were generated by transferring 10^4 naive CD45.1 cells to naive mice, and 1 d later the mice were infected with 10^5 LM-OVA. CD8 T cells were enriched by positive selection using autoMACS (Miltenyi Biotec). CD45.1+ memory CD8 T cells were FACs sorted from enriched CD8 cells using a FACsAria (BD Biosciences). For experiments, naive mice received 5 × 10^5 sorted memory CD45.1 OT-I cells and 24 h later were infected with 10^5 LM-OVA.

Results

CCR7 is important for naive CD8 T cells migration into the splenic T cell zones

To confirm that CCR7 regulates naive CD8 T cells trafficking to the splenic T cell zones, we adoptively transferred 0.5 × 10^6 CD45.1+ WT, CCR7−/−, or CD2-CCR7 OT-I cells into uninfected congenic CD45.2− recipient mice i.v. Mice were sacrificed 2 d
after transfer and the spleens were analyzed by flow cytometry and confocal microscopy. CD2-CCR7 OT-I cells expressed higher levels of CCR7 on their surface compared with WT OT-I cells, and CCR7<sup>−/−</sup> OT-I cells failed to express CCR7 (Supplemental Fig. 1A). Within the spleen, WT OT-I cells were observed in both WP and RP, whereas the CCR7<sup>−/−</sup> OT-I cells were located primarily in the RP (Fig. 1, pie charts). As expected, CD2-CCR7 OT-I cells were located exclusively in the T cell zones (Fig. 1). The analysis of the peripheral tissues revealed (Supplemental Fig. 1B) that CD2-CCR7 OT-I cells migrated and were retained exclusively in the spleen and lymph nodes, and not in any other peripheral nonlymphoid organs or in circulation. CCR7<sup>−/−</sup> OT-I cells were present in the splenic RP and nonlymphoid tissues but not in the lymph nodes. As expected, WT OT-I CD8<sup>+</sup> T cells were detected in all organs tested (Supplemental Fig. 1B).

CCR7<sup>−/−</sup> OT-I cells enter the T cell zones early after infection; however, CCR7 migratory cues are required for ordered effector CD8<sup>+</sup> T cell egress from the splenic T cell zones

To visualize the early activation of Ag-specific CD8<sup>+</sup> T cells, we transferred 0.5 × 10<sup>6</sup> WT, CCR7<sup>−/−</sup>, or CD2-CCR7 OT-I cells into naive mice that were infected i.v. with 10<sup>5</sup> CFU actA<sub>2</sub>LM-OVA (hereafter referred to as LM-OVA). Spleens were analyzed at 10, 24, 48, and 72 h postinfection (PI) for the presence of OT-I cells. As shown in Fig. 2A, in contrast to WT OT-I cells, virtually every CCR7<sup>−/−</sup> OT-I cell was either present in MZ or RP at 10 h (orange arrows). By 24 h PI, WT OT-I cells were still localized to the T cell zones (blue arrows); however, CCR7<sup>−/−</sup> OT-I cells appeared to migrate from the MZ/RP to the B cell zones (white arrowheads), and surprisingly several cells were also detected in the T cell zones (blue arrows). By 48 and 72 h PI, the migratory properties of WT and CCR7<sup>−/−</sup> OT-I cells were strikingly distinct. At 48 h PI, most WT OT-I cells were exclusively positioned in the T cell zones (Fig. 2). In contrast, a large percentage of CCR7<sup>−/−</sup> OT-I cells appeared to have undergone peripheral migration within the WP and were primarily located in the B cell zones or at the borders of the B–T cell zones, whereas a smaller percentage (∼18%) was located in the RP. Additionally, small clusters of CCR7<sup>−/−</sup> OT-I cells could be observed in the RP, indicating that Ag was also being presented to T cells in the splenic RP (boxes). By 72 h PI, WT OT-I cells had undergone a dramatic expansion and could be observed exiting the T cell zones via the bridging channels (Fig. 2A) (17). However, CCR7<sup>−/−</sup> OT-I cells had proliferated, albeit at a reduced rate compared with WT OT-I cells, and were localized in the T and B cell zones. Additionally, a greater number of CCR7<sup>−/−</sup> OT-I cells were now in the RP. Thus, these data show that in the absence of CCR7 the migratory cues followed by Ag-specific CD8<sup>+</sup> T cells following infection are
dramatically distinct when compared with the migratory patterns of WT CD8 T cells. Although CCR7−/− OT-I cells migrated to the T cell zones from the RP, they failed to use the bridging channels to either enter (24 h PI) or exit (48–72 h PI) the splenic T cell zones. In fact, CCR7−/− OT-I cells migrated through the B cell zones and most of them primarily remained in the periphery of the T cell zones and appeared to exit the T cell zones with greater alacrity when compared with WT OT-I cells. The unique localization pattern of CCR7−/− OT-I cells suggests that even when CCR7 is downregulated on activated T cells, some level of CCR7 signaling continues to guide the entry and egress kinetics of T cells in the spleen. These data clearly show that even in the absence of CCR7, OT-I cells entered the T cell zones; however, most of these T cells stayed in the periphery of the parietal lymphoid sheath (PALS) or entered the B cell zones. The chemokine receptor CXCR5 has been shown to allow CD8 T cell entry into reactive lymph nodes even in the absence of CCR7 (18). Furthermore, CXCR3 has been implicated in the peripheral localization of central memory CD8 T cells within the lymph nodes (19, 20). However, its role in trafficking of CD8 T cells in the spleen is not well understood. Moreover, the chemokine receptor CXCR5 is required for entry of T cells (particularly CD4 T cells) to the B cell zones where the ligand CXCL13 is produced. Thus, it is likely that in the absence of CCR7, CXCR3 and CXCR5 may play a role in allowing for the distinct trafficking pattern followed by CCR7−/− OT-I cells. Indeed at 2 d PI both CXCR3 and CXCR5 are upregulated in both WT and CCR7−/− OT-I cells (Supplemental Fig. 2). However, in the case of WT cells, CCR7-mediated cues may yet remain dominant and prevent the disordered peripheral migration of WT CD8 T cells into the B cell zones, as seen with CCR7−/− OT-I cells.

**CCR7-mediated migratory cues determine the magnitude and the differentiation phenotype of responding CD8 T cells**

We next determined whether the altered migration and localization pattern of CCR7−/− OT-I cells within the splenic tissue affects the magnitude of effector CD8 T cell expansion and/or the differentiation program of the responding effector CD8 T cells following *L. monocytogenes* infection. We reasoned that WT OT-I cells will be primed primarily in the splenic T cell zones and will remain in the splenic T cell zones for the appropriate length of time. Conversely, CCR7−/− CD8 T cells will likely be primed mainly in the splenic RP, and those T cells that do gain access to the T cell zones will exhibit a disordered egress pattern characterized by premature exit from the T cell zones. Additionally, CD2-CCR7 OT-I cells will be primed exclusively in the T cell zones. Therefore, we adoptively transferred 10^7 naive WT, CCR7−/−, or CD2-CCR7 OT-I cells in mice. Twenty-four hours later, these mice were infected with LM-OVA. At days 5 and 7 PI the spleen from each mouse was cut in two equal halves, with one half used for imaging studies and the other for flow cytometric comparison. As shown in Fig. 3A, at both 5 and 7 d PI, WT OT-I cells were located in both WP and RP, and CCR7−/− OT-I cells were found largely in RP of the spleen, whereas CD2-CCR7 OT-I cells were strikingly confined to the T cell zones and failed to exit the splenic WP. Although CCR7−/− OT-I cells expanded equally at 5 d PI (data not shown), by 7 d the expansion of these cells was significantly reduced when compared with WT or CD2-CCR7 OT-I cells (Fig. 3B). The observed reduced expansion of CCR7−/− OT-I cells in the spleen was not due to increased migration of these cells in the peripheral tissues, because we did not find increased numbers of these cells in the lungs or liver (Supplemental Fig. 3A); the expansion in the peripheral organs of CCR7−/− OT-I cells was also significantly decreased compared with WT OT-I cells. Interestingly, the percentage of CD2-CCR7 OT-I cells in the peripheral tissue was severely reduced, which was likely due to their inability to migrate out of the spleen. Although at the peak of the immune response the expansion of CCR7−/− OT-I cells was significantly decreased compared with WT OT-I cells, the percentage of CCR7−/− CD8 T cells capable of secreting IFN-γ was equal to WT or CD2-CCR7 OT-I cells (Fig. 3C). To determine whether the initial expansion and replication of OT-I cells in the absence of CCR7 contribute to their poor expansion, we evaluated the ability of each OT-I cell population to proliferate early after infection. Indeed, the initial expansion of CCR7−/− OT-I cells was compromised when compared with WT or CD2-CCR7 OT-I cells (Supplemental Fig. 3B) as judged by CFSE loss at day 2 PI. However, 24 h later (at day 3 PI) virtually all groups of T cells present in the spleen exhibited comparable loss of the CFSE stain. Similarly, BrdU incorporation at day 3 PI was comparable for all three types of OT-I cells (Supplemental Fig. 3C). There are many factors that affect the balance between short-lived effector cell (SLEC; KLRG1^high^CD127^low^) and memory precursor effector cell (MPEC; KLRG1^low^CD127^high^) formation at early time points after infection. These include proinflammatory cytokines and strength of stimuli and its duration. We wanted to analyze whether priming location and the subsequent migratory cues of CD8 T cells in different splenic compartments can have an effect on effector T cell differentiation. To this end, the differentiation profile of effector CD8 T cells at earlier time points after infection was analyzed by evaluating the expression of KLRG1 and CD127. Interestingly, significantly more OT-I cells that were not directed by CCR7-mediated migratory cues (CCR7−/− OT-I) and are primarily located in the RP (at 5 and 7 d PI) had differentiated into SLECs and less MPECs in comparison with WT or CD2-CCR7 OT-I cells (Fig. 3D).

**CCR7 migratory cues during the priming phase are required for proper memory CD8 T cell generation**

Our data thus far clearly showed that CD8 T cell activation and trafficking were dramatically altered during the effector phase of the antimicrobial immune response. Thus, in the next set of experiments we sought to determine whether quantity and quality of memory CD8 T cell were also affected by the absence of T cell–intrinsic CCR7. To this end, we first analyzed the localization of all three groups of memory OT-I cells. As shown in Fig. 4A, early memory OT-I cells in mice sacrificed at day 30 PI were localized in distinct areas of the spleen. WT OT-I memory cells were widely distributed throughout the different splenic compartments; however, most were located in the RP and B cell zones (yellow arrows), whereas a smaller percentage was in T cell zones (blue arrows). CCR7−/− OT-I memory cells were exclusively found in the splenic RP (orange arrows), whereas CD2-CCR7 memory OT-I cells were exclusively located in the T cell zones. The expression of CCR7 on these early WT memory cells was low (data not shown).

Flow cytometric analysis of splenocytes revealed that mice that received WT and CD2-CCR7 OT-I cells harbored a healthy population of memory CD8 T cells. However, memory T cell generation was severely compromised in mice that received CCR7−/− OT-I cells (Fig. 4B). To confirm that the reduced memory CCR7−/− OT-I numbers was not due to the increased migration of these cells to the peripheral organs, we assayed various organs for OT-I cells and found that the numbers of CCR7−/− OT-I cells in the indicated organs was similarly low (Supplemental Fig. 4A). Interestingly, the number of CD2-CCR7 OT-I cells was also dramatically reduced in nonlymphoid organs such as the liver and lung, suggesting that after activation the failure to downregulate CCR7 in these cells prevented the migration of effector cells from lymphoid
organs to the periphery. Although the number of memory CCR7
2
2
OT-I cells was dramatically reduced in the spleen, they were
functionally as capable of secreting cytokines as the WT memory
OT-I cells (Fig. 4C). These data suggest that CCR7-directed mi-

FIGURE 2. CCR7−/− OT-I cells enter the T cell zones early after infection; however, CCR7 migratory cues are required for ordered effector CD8 T cells egress from the splenic T cell zones. (A) Naive mice received 0.5 million CCR7−/− OT-I and WT OT-I cells and were infected next day with 10⁵ LM-OVA. Mice were sacrificed at 10, 24, 48, and 72 h PI and spleens were harvested for confocal microscopy. (B) Pie charts show percentages of OT-I cells (of total OT-I cells) in different splenic compartments. Light blue arrows point to OT-I CD8 T cells inside the T cell zone whereas orange arrows point to OT-I CD8 T cells in RP. White arrows represent the OT-I cells present in B cell zone. The data are representative of at least two to three experiments with n = 3 mice for each group. Scale bars, 50 μm. B, B cell zone; BC, bridging channel; T, T cell zone.
gration and localization early during the primary phase of T cell activation are essential for adequate formation of a memory T cell pool.

Expression of CCR7 and localization of memory CD8 T cells within the spleen do not affect the magnitude of recall responses

Because memory CCR7−/− OT-I cells were localized primarily in splenic RP and memory CD2-CCR7 OT-I cells were exclusively positioned in splenic T cell zones, we next wanted to determine how CCR7 expression and the localization of memory CD8 T cells in different compartments of the spleen would affect recall responses after challenge infection. Mice that were transferred with the three different types of OT-I cells and infected with LM-OVA 30 d earlier were rechallenged with LM-OVA and spleens were imaged at 5 d after recall. As shown in Fig. 5A, WT OT-I cells were observed both in the splenic RP and WP, with most located in the MZ or RP. In contrast, CCR7−/− OT-I cells were primarily located in the splenic RP. In remarkable contrast, CD2-CCR7 OT-I cells were completely confined to the T cell zones of the spleen (Fig. 5A). Interestingly, at 5 d after recall the expansion of all three memory OT-I cells was robust (Fig. 5B); however, the number of CCR7−/− OT-I cells was slightly reduced (∼2-fold) compared with WT and CD2-CCR7 OT-I cells. This reduction in responding CCR7−/− OT-I memory cells was likely due to the reduced numbers of memory cells generated in CCR7−/− mice (∼20-fold reduction; Fig. 4B). To further confirm that the reduction in memory CCR7−/− OT-I cell expansion was not due to increased migration of these OT-I cells to peripheral organs, we analyzed these organs for the presence of CCR7−/− OT-I cells and found that their numbers were similarly reduced when compared with the WT OT-I cells (Supplemental Fig. 4B).

To definitively understand how localization of memory CD8+ T cells within the spleen influences the recall response, we transferred equal numbers of CCR7−/−, WT, or CD2-CCR7 memory OT-I cells (5 × 10⁴ cells) to naive mice and challenged the naive

**FIGURE 5.** CCR7-mediated migratory cues determine the magnitude, localization, and the differentiation phenotype of responding CD8 T cells. Naive mice received 10⁴ different types of OT-I cells 1 d prior to infection. Spleens were harvested at days 5 and 7 PI and divided into two halves. (A) One half of the spleens were sectioned for microscopic analysis. Light blue arrows point to OT-I CD8 T cells inside the T cell zone, whereas orange arrows point to OT-I CD8 T cells in RP and MZ. Scale bars, 50 μm (top center, top right, bottom left), 100 μm (top left, bottom center, bottom right). (B) The other half of the spleen was analyzed by flow cytometry at day 7 PI. (C) Mice received 10⁵ of the indicated types of OT-I cell 1 d prior to infection. Spleens were harvested at day 7 PI, and lymphocytes were processed for intracellular cytokine staining. (D) Spleens of mice that received the different types of OT-I cells were analyzed for SLEC (KLRG1highCD127low) and MPEC (KLRG1lowCD127low) populations at days 5 and 7 PI. The bar graph shows the percentages of indicated population of total OT-I cells. The data are representative of at least two to three experiments with n = 3–4 mice for each group. Standard error of mean is shown with *p < 0.05, **p < 0.001, ***p < 0.001. B, B cell zone; BC, bridging channel; T, T cell zone.
mice 1 d later with LM-OVA. The spleens were isolated at day 5 PI, and the OVA-specific memory T cell response was analyzed. Interestingly, CCR7−/− OT-I cell expansion was as robust as the WT or CD2-CCR7 OT-I cells (Fig. 5C), suggesting that the CCR7-dependent differential localization of memory CD8+ T cells within the spleen did not affect the recall response. This was in contrast to the primary response where proper T cell expansion was heavily dependent on CCR7 migratory cues. We also analyzed the differentiation pattern of recalled memory OT-I cells. SLEC formation of recalled OT-I cells was not affected by the differential expression of CCR7 (Fig. 5D), which was also in contrast to the primary response.

Following reinfection, early memory CD8 T cells enter the splenic T cell zones independently of CCR7 through the B cell zones

We found that CD8 T cells enter the splenic T cell zones independently of CCR7 at earlier time points after primary infection. Because early memory CD8 T cells also have lower surface expression of CCR7, we wanted to know whether early WT memory OT-I cells or CCR7−/− OT-I memory cells can enter the T cell zones through the B cell zones. Equal numbers of WT or CCR7−/− memory OT-I cells were purified from mice infected 30 d previously and transferred into naive mice that were recalled with LM-OVA. The spleens were excised at day 2 PI and sections were analyzed for OT-I cells by confocal microscopy. As shown in Fig. 6, even memory CCR7−/− but also WT OT-I memory CD8 T cells could be observed in the B cell zones. These results suggested that CCR7 low-memory CD8 T cells can enter the T cell zones independently of CCR7 and that the trafficking pattern of both WT and CCR7−/− memory CD8 T cells is similar, which is in contrast to what was observed early after primary infection.

Discussion
In the present study, we investigated how CCR7 regulates naive, effector, and memory CD8 T cell migration after bacterial infection in the spleen. Although the role of CCR7 in regulating CD8 T cell trafficking into the lymph nodes has been extensively studied (12, 21), less is known about its effect on T cell migration in the spleen. Because i.v. inoculation of mice with L. monocytogenes results in an infection that is largely confined to the spleen (22), we were able to specifically visualize CD8 T cell migration within different splenic compartments. As expected, CCR7 was required for the localization of naive T cells in the splenic T cell zones.
Initially we reasoned that by using adoptive transfer of CCR7 transgenic and deficient OT-I CD8 T cells we would be able to exploit the unique positioning of these Ag-specific naive CD8 T cells to ask how CD8 T cell activation is affected when these cells are exclusively primed in T cell zones or in the RP of the spleen. After infection, CD8α+ DCs transport *L. monocytogenes* to the splenic T cell zones (2, 23), and therefore it is thought that CD8 T cells encounter Ag and are subsequently primed within T cell zones. Thus, we hypothesized that CCR72/2 CD8 T cells that fail to enter the T cell zones would be poorly activated. However, to our surprise, our results demonstrated that CCR72/2 OT-I cells in fact migrated to the WP at early time points after infection. Because the naive CCR72/2 CD8 T cells are primarily located in the splenic RP, these cells likely migrated to the WP after infection and following the transport of *L. monocytogenes* to the T cell zones. In this respect, the infected splenic T cell zone represents a “reactive” area where bacteria-induced inflammation likely results in the production of several different chemokines other than CCL19/21 that attract the CCR72/2 OT-I cells to migrate to the splenic T cell zones. The identity of this chemokine is presently unknown, but CXCR3 may be a possible candidate (18). However, the main CXCR3 ligand CXCL9 is not expressed in the T cell zones of the spleen, but it is primarily produced in the MZ (9). These data suggest that CXCR3 may not be responsible for allowing CCR72/2 OT-I cells to migrate to the T cell zones. Thus, we hypothesize that CCR72/2 CD8 T cells migrate to the reactive T cell zones in the spleen where most of these T cells are primed. However, we cannot exclude the possibility that a small proportion of these cells encountered Ag very early within hours after infection in the RP before expanding and exiting the spleen via the RP. Our data clearly showed that even in the absence of CCR7, OT-I CD8 T cells migrated to the splenic T cell zones where they were primed; however, after infection, the migratory and activation patterns of OT-I cells in the absence of CCR7 were dramatically distinct when compared with WT OT-I cells. Our data clearly demonstrated that although the entire population of the transferred CCR72/2 OT-I cells in the spleen had been primed (as judged by CD69 expression at 24 h PI; data not shown), these cells...
failed to expand with the same magnitude as did the WT or CD2-CCR7 OT-I cells. This result could be partially explained by the fact that a greater percentage of CCR7$^{-/-}$ OT-I cells exhibited the SLEC phenotype and thus were terminally differentiated. Interestingly, at early time points (day 3 PI), both WT and CCR7$^{-/-}$ OT-I cells exhibited comparable CFSE dilution, and at 5 d PI the number of WT and CCR7$^{-/-}$ OT-I cells in the spleen was similar. However, by 7 d PI the number and frequency of CCR7$^{-/-}$ OT-I cells in the spleen was dramatically reduced. This could be due to a combination of two reasons: because greater frequencies of CCR7$^{-/-}$ OT-I cells are terminally differentiated, these cells are likely undergoing early apoptosis. Moreover, the failure to expand adequately is likely also related to the inability of CCR7$^{-/-}$ OT-I cells to be sequestered within the T cell zones long enough to receive the adequate amount of signals from APCs, leading to poor proliferation and survival. As noted earlier, CCR7$^{-/-}$ OT-I cells entered the T cell zones; however, most of these T cells stayed in the periphery of the PALS or entered the B cell zones. These cells expressed high levels of CXCR3 and CXCR5, and thus it is likely that in the absence of CCR7, both CXCR3 and CXCR5 may play dominant roles in mediating the disordered peripheral positioning and migration of CCR7$^{-/-}$ OT-I cells through the B cell zones. This is in contrast to the protracted sequestration of WT OT-I cells in the splenic T cell zones and the ordered egress of WT OT-I cells from the T cell zones into the RP via the bridging channels (17) following infection. Thus, our study clearly demonstrated that although the cell surface expression of CCR7 is reduced on WT OT-I cells after activation, some level of tonic CCR7-mediated cues may yet remain dominant and prevent the disordered peripheral migration of WT CD8 T cells into the B cell zones, as observed with CCR7$^{-/-}$ OT-I cells. Moreover, the migratory and localization cues provided by CCR7 are essential for the adequate expansion and differentiation of CD8 T cells early after infection in the spleen.

Interestingly, CCR7-mediated signals were not important for the proper expansion of memory CD8 T cells. This may be related to the possibility that memory CD8 T cells exhibit a lower threshold of activation and do not require protracted sequestration in the splenic T cell zones after rechallenge for proper expansion and differentiation. Moreover, even WT memory CD8 T cells at 30 d PI (early memory CD8 T cells) fail to express high levels of CCR7; in this respect, they are similar to CCR7$^{-/-}$, and thus they appear to exhibit similar trafficking patterns that are very distinct from primary effector CD8 T cells.

In summary, our study showed that alterations in CCR7 expression can dramatically influence localization, migration, and activation of Ag-specific CD8 T cells in the spleen following a bacterial infection. Thus, our results advance the understanding of how CCR7-mediated guidance cues affect the distribution and localization of CD8 T cells within the splenic microenvironment and how these guidance cues shape the differentiation and function of effector and memory CD8 T cells following infection.

**Acknowledgments**

We thank Quynh-Mai Pham and Leigh Maher for assistance in performing experiments, and Dr. Evan Jellison in the University of Connecticut Health Flow Cytometry Facility and the Center for Cell Analysis and Modeling for help with imaging experiments.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


