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Differential Localization of Effector and Memory CD8 T Cell Subsets in Lymphoid Organs during Acute Viral Infection

Yong Woo Jung,* Rachel L. Rutishauser,* Nikhil S. Joshi,* Ann M. Haberman, † and Susan M. Kaech*‡

It is unclear where within tissues subsets of effector and memory CD8 T cells persist during viral infection and whether their localization affects function and long-term survival. Following lymphocytic choriomeningitis virus infection, we found most killer cell lectin-like receptor G1 (KLRG1)hiIL-7Rlo effector and memory cells, which are long-lived and high proliferative capacity, in the T cell zone of the spleen. In contrast, KLRG1loIL-7Rhi cells, which appear terminally differentiated and have shorter life spans, were exclusively localized to the red pulp. KLRG1loIL-7Rhi T cells homed to the T cell zone using pertussis toxin-sensitive chemokine receptors and appeared to contact gp38+ stromal cells, which produce the chemokines CCL19 and CCL21 and the T cell survival cytokine IL-7. The transcription factors T-bet and B lymphocyte-induced maturation protein-1 controlled effector CD8 T cell splenic migration. Effector CD8 T cells overexpressing T-bet homed to the red pulp, whereas those lacking B lymphocyte-induced maturation protein-1 homed to the T cell zone. Upon memory formation, CD62L+ memory T cells were predominantly found in the T cell zone, whereas CD62L− cells were found in the red pulp. Thus, effector and memory CD8 T cell subset localization within tissues is linked to their differentiation states, and this may identify anatomical niches that regulate their longevity and homeostasis. The Journal of Immunology, 2010, 185: 000–000.

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The online version of this article contains supplemental material.

Abbreviations used in this paper: B, B cell; Blimp-1, B lymphocyte-induced maturation protein-1; BM, bone marrow; DC, dendritic cell; FRC, fibroblastic reticular cell; KLRG1, killer cell lectin-like receptor G1; KO, knockout; LCMV, lymphocytic choriomeningitis virus; LN, lymph node; p.i., postinfection; PTX, pertussis toxin; RP, red pulp; RV, retrovirus; S1PR5, sphingosine 1-phosphate receptor 5; T, T cell; TCM, central memory T; TEM, effector memory T; WP, white pulp; WT, wild-type.

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IL-15 is produced by cells of the hematopoietic system, such as dendritic cells (DCs) and macrophages (2, 7, 8). IL-15 can be induced by type I IFNs during infection (9, 10); however, under resting conditions, basal levels of IL-15 help to replenish the pool of memory CD8 T cells by driving a slow, yet steady, rate of turnover (2). Although it is starting to be better understood how memory T cells receive these cytokines in vivo (6, 8, 11), it remains unclear whether specialized microenvironments or niches exist in tissues that optimally promote memory T cell development, survival, and homeostasis. In addition, it is not known how the trafficking of effector and memory T cells to such sites is regulated.

Following most acute infections, memory CD8 T cells descend from a heterogenous pool of effector CD8 T cells, composed of numerous subsets associated with different functional capacities and long lives. Some subsets have been characterized based on the differential expression of surface receptors. For example, it was noted during several acute systemic infections, such as with lymphocytic choriomeningitis virus (LCMV) and Listeria monocytogenes, that the majority of effector CD8 T cells express large amounts of killer cell lectin-like receptor G1 (KLRG1) and small amounts of IL-7R and are functional CTLs that produce IFN-γ and TNF-α (12–17). However, the KLRG1loIL-7Rhi cell subset predominantly contains terminal effector cells, which have a reduced ability to produce IL-2, and proliferate in response to Ag or homeostatic cytokines. Although these cells are highly dependent on IL-15, they have a finite lifespan and generally do not develop into long-lived memory CD8 T cells. Conversely, a smaller effector cell subset exists that displays the inverse expression pattern of KLRG1 and IL-7R (i.e., KLRG1hiIL-7Rhi). Relative to the terminally differentiated KLRG1loIL-7Rhi cells, the KLRG1hiIL-7Rhi cells are enriched with polyfunctional CTLs that coproduce IFN-γ, TNF-α, and IL-2. These memory precursor cells respond to IL-7 and IL-15, are longer-lived, express more Bcl-2, and, as they mature into memory CD8 T cells, exhibit cardinal memory cell properties, such as the ability to self-renew and proliferate after secondary infection. In addition to these two subsets, other subpop-
ulations of effector cells can be identified. For instance, cells that express KLRG1 and IL-7R also populate the memory CD8 T cell pool and tend to have an intermediate life span and proliferative capacity relative to the other two subsets (12, 18).

Like the effector CD8 T cell population, the memory CD8 T cell population is heterogeneous and consists of subsets that vary in function and anatomical location. In addition to differential expression of KLRG1 and IL-7R, memory CD8 T cells can be distinguished based on CD27, CD28, CD62L, CCR7, and CD103 expression (19–21). In general, central memory T (TCM) cells express increased amounts of CD26L, CCRL7, CD27, and CD28 and preferentially localize to the spleen and LNs. TCM cells tend to produce more IL-2 and have robust proliferative responses upon secondary infection. In contrast, effector memory T (TEM) cells express lower amounts of LN-homing molecules and costimulatory receptors and increased amounts of cytotoxic proteins. Consequently, TEM cells are primarily found in the blood and non-lymphoid tissues (e.g., liver and lung) (1, 14, 22, 23). In addition to TCM cells, resident memory cells have been found in the skin, brain, and intestine and seem to persist in these tissues long term without apparent recirculation (24–26). Understanding the heterogeneity of effector and memory CD8 T cell populations is important for understanding the relative functional contributions of different subsets to short- and long-term protective immunity.

The formation of these different effector and memory CD8 T cell subsets can be influenced by multiple factors. For example, increased intensity or duration of inflammation and infection enhances the formation of greater numbers of terminally differentiated KLRG1hiIL-7Rlo effector cells, and IL-12 and IL-2 are important signals involved in their differentiation (12, 27–33). These transcription factors also regulate effector and memory CD8 T cell trafficking. For example, increased expression of T-bet and B lymphocyte-induced maturation protein-1 (Blimp-1) drives the formation of terminally differentiated KLRG1hiIL-7Rlo effector cells (12, 34, 35). Interestingly, T-bet (encoded by Tbx21) and Blimp-1 (encoded by Prdm1) expression can be regulated by IL-12 and IL-2, demonstrating a tight association between inflammatory cytokines and the regulation of effector and memory cell potential within activated CD8 T cells. In contrast, increased expression of the transcription factors comesdolirini and Bcl-6 are associated with memory precursor cell states, but their requirement in these cells is unknown (32, 34, 35). In the mature memory CD8 T cell population, transcription factors, such as Id2 and Blimp-1, limit TCM cell formation; conversely, increased Bcl-6 expression augments their formation (34–37). These transcription factors also regulate effector and memory CD8 T cell trafficking. For example, Blimp-1–deficient effector cells express more CCR7 mRNA compared with wild-type effector T cells and tend to localize preferentially to lymphoid tissues (34, 35). Differences in chemokine receptor expression between the distinct subsets may permit differential localization in tissues where important survival factors are received.

In this study, we aimed to better understand the development and maintenance of effector and memory T cell subsets in vivo by investigating their localization in the spleen following LCMV infection. Our data demonstrate that KLRG1hiIL-7Rlo and KLRG1loIL-7Rhi CD8 T cells inherently reside in largely distinct areas of the spleen; KLRG1loIL-7Rhi cells were observed in the white pulp (WP) and red pulp (RP), whereas KLRG1hiIL-7Rlo cells were found exclusively in the RP. The differential expression of chemokine receptors, such as CCR7, between the two subsets was also observed and likely contributes by facilitating migration and interactions with the appropriate cytokine-producing cells that regulate their survival and homeostasis. These data suggest that the longevity and maintenance of different subsets of effector and memory T cells depend on receiving different signals from the cells with which they make contact in different anatomical regions within tissues.

Materials and Methods

Mice and infections

C57BL/6 (B6) mice were obtained from the National Cancer Institute (Frederick, MD), Ly5.1+ P14 TCR transgenic mice and the generation of P14 chimeric mice via transfer of 25,000 naive P14 CD8+ T cells into B6 mice were described previously (12, 35). Mice were infected with 2 × 10⁶ PFU LCMV-Armstrong i.p. All animal experiments were carried out according to approved Institutional Animal Care and Use Committee protocols.

Immunofluorescent microscopy

At desired time points postinfection (p.i.), spleens were isolated and embedded in OCT compound (Sakura, Torrance, CA). Tissue blocks were frozen in isopentane (Sigma-Aldrich, St. Louis, MO) chilled with dry ice. Tissues containing GFP-expressing cells were fixed with 4% paraformaldehyde before freezing. Eight-micrometer sections were cut using a cryostat, air dried, and fixed with cold acetone. Sections were stained with 1–5 μg/ml fluorescein isothiocyanate- or biotin-labeled Abs specific for CCL21 (R&D Systems, Minneapolis, MN), gp38 (Developmental Studies Hybridoma Bank, Iowa City, IA), CD4, B220, F4/80, Ly5.1, Ly5.2, CXL2L, KLRG1, or IL-7R (eBioscience, San Diego, CA). Biotinylated Abs were visualized with Alexa 568-conjugated streptavidin (Invitrogen, Carlsbad, CA) or, in some cases, were amplified using a tyramide signal-amplification kit (PerkinElmer, Waltham, MA). Images were captured on an Olympus BX-40 microscope with a SPOT-RT Slide (Scallanetics, Rockville, MD) digital camera. To quantitate the number and phenotypes of LCMV-specific CD8 T cells within different splenic regions, equally sized boxed regions were applied to the T cell zone, B cell zone, or RP for each tissue section, and P14 cells were enumerated and scored for Ly5.1, IL-7R, and KLRG1 expression using ImageJ software (National Institutes of Health, Bethesda, MD) and the “ColocalizeRGB” plug-in.

In vivo migration assay

P14 GFP+ KLRG1hiIL-7Rlo and KLRG1loIL-7Rhi cells were sorted by FACS on day 9 p.i. Equal numbers of cells of each subset were transferred into naive mice and analyzed by fluorescent microscopy 2 d later. For pertussis toxin (PTX) treatment, sorted cells were incubated with RPMI 1640 alone or RPMI 1640 containing PTX (Sigma-Aldrich) for 1 h at 37°C and washed twice before transferring to B6 animals.

Retrovirus constructs and transduction

P14 splenocytes activated with gp38 peptide were transfected with MigR1 control or T-bet retrovirus (RV), as described previously (38). Mice containing RV-transduced P14 CD8 T cells were sacrificed at day 6 posttransduction, and GFP+ P14 T cells were analyzed by immunofluorescent microscopy.

Real-time PCR analyses

Four effector and memory cell populations were isolated by FACS: day 9 p.i. P14 CD8 T cells, KLRG1loIL-7Rhi cells, KLRG1hiIL-7Rlo cells, and memory cells isolated 6 mo after LCMV infection. Additionally, naive P14 CD8 T cells were prepared by FACS. Total RNA was extracted by the TRIzol method (Invitrogen), and cDNA was synthesized using SSRTII for Ly5.1, IL-7R, and KLRG-1 expression using ImageJ software (National Institutes of Health, Bethesda, MD) and the “ColocalizeRGB” plug-in.

Transwell-migration assay

Cells were tested for transmigration across uncoated 5-μm Transwell filters (Corning, Lowell, MA) for 3 h to CCL19 (Sigma-Aldrich), CXCL12 (PeproTech, Rocky Hill, NJ), or medium in the bottom chamber and then enumerated by flow cytometry.

Statistical analysis

Standard two-tailed t tests were used for all statistical calculations. All error bars and variances represent SEM.
Results

Subsets of effector and memory T cells are differentially localized in the spleen

To investigate the localization of subsets of effector and memory T cells within the spleen, we generated P14 chimeric mice by transferring small numbers of Ly5.1+ P14 transgenic CD8 T cells, which recognize the D\textsuperscript{b}GP\textsubscript{33-41} epitope of LCMV, into Ly5.2+ recipient mice and infected these mice with LCMV. On days 8, 15, and 30 p.i., spleens were isolated and cut in half. Single-cell suspensions were prepared from one half of the spleen; stained with Abs to CD8, Ly5.1, IL-7R, KLRG1, and other proteins; and examined using flow cytometry (Fig. 1, contour plots atop each column). In agreement with previous reports, most effector cells were KLRG1\textsuperscript{hi}IL-7R\textsuperscript{lo} on day 8 p.i., and the frequency of this subset gradually decreased between days 15 and 30 p.i. It was noted previously that the reduction in KLRG1\textsuperscript{hi}IL-7R\textsuperscript{lo} cells occurs primarily as a result of cell death, rather than conversion to a KLRG1\textsuperscript{lo}IL-7R\textsuperscript{hi} phenotype (12, 39).

The other half of the spleen was used to make frozen serial sections that were analyzed using four-color immunofluorescent microscopy to determine the localization of the virus-specific effector and memory CD8 T cells. To identify regions of interest, the first serial section was stained with CD4 (T cell zone), B220 (B cell zone), and F4/80 (RP). The second section was stained with B220 (data not shown), Ly5.1 (P14 cells), IL-7R, and KLRG1 to identify the location of the different CD8 T cell subsets (Fig. 1). Based on the Ly5.1 staining, P14 cells were found in the T cell zone, RP, and B cell zone on days 8, 15, and 30 p.i. (Fig. 1A–C). Next, we counted the number of cells within equally sized areas in the T cell zone, B cell zone, and the RP. The density of the Ag-specific CD8 T cells

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in the spleen correlated with the increase and decrease in the frequency of the virus-specific CD8 T cells over the course of infection (Fig. 1D). For example, >1000 P14 cells per mm² were found in the T cell zone and the RP on day 8 p.i., but this decreased to <200 per cm² at days 15 and 30 p.i.

Next, we examined where KLRG1hi or IL-7Rhi LCMV-specific effector and memory CD8 T cells were located in the spleen following infection. At day 8 p.i., ~60–80% of the LCMV-specific KLRG1loIL-7Rhi memory precursor cells were found in the T cell zone, with the remainder located in the RP (Fig. 1A, 1E). In contrast, terminal KLRG1hiIL-7Rlo effector CD8 T cells were found exclusively in the RP (Fig. 1A, 1F). As effector cells contracted and memory CD8 T cells began to form between days 15 and 30 p.i., the localization of the different subsets remained similar to that found at day 8 (Fig. 1B–F), implying that the homing of these subsets is largely intrinsic to the differentiation state of the CD8 T cell rather than the changes in the environment caused by infection. To confirm this conclusion, we also analyzed the localization of these populations on day 6 p.i., when virus is still present and the splenic microenvironment is more inflamed (Supplemental Fig. 1). Similar to the other time points analyzed, the KLRG1loIL-7Rhi effector CD8 T cells lose the ability to home to the T cell zone as soon as they form, whereas most KLRG1loIL-7Rhi cells retain this ability. We also found that differential homing of effector and memory T cell subsets occurred in the LN (Supplemental Fig. 2). In this case, KLRG1loIL-7Rhi cells were found in the T cell zone, B cell zone, and medullary area, whereas KLRG1hiIL-7Rlo CD8 T cells were exclusively localized in the medullary area. These data show that virus-specific CD8 T cells are localized within the lymphoid organs according to their differentiation state.

KLRG1hiIL-7Rlo effector T cells actively home into the T cell zone using their chemokine receptors

To test whether effector cells actively home to these distinct areas of the spleen, we made chimeric mice with P14 CD8 T cells that express GFP and infected them with LCMV. On day 9 p.i., subsets of effector cells from the spleen were purified by FACS, based on their expression of IL-7R and KLRG1. These cells were transfected i.v. into uninfected B6 animals; 2 d later, their locations in the spleen were analyzed by immunofluorescent microscopy. The results were very similar to those found in the unmanipulated animals (Fig. 1). Although KLRG1loIL-7Rhi effector CD8 T cells migrated into the WP and RP, KLRG1hiIL-7Rlo effector cells were exclusively found in the RP (Fig. 2A, 2B). The fraction of each donor cell population found within the WP or RP was calculated; this demonstrated that the KLRG1loIL-7Rlo cells migrated into the WP and RP at a roughly 60:40 ratio, whereas >90% of KLRG1loIL-7Rlo cells were present in the RP (Fig. 2C).

We further studied whether migration to each region required chemokine receptor-dependent signaling pathways. Using the technique described above, we isolated subsets of effector cells and treated them with media alone or media containing PTX, which inhibits the signaling of most G protein-coupled chemokine receptors (40, 41). Naive P14 CD8 T cells were used as a control, because their migration into the T cell zone is impaired by PTX treatment (42). Naive and KLRG1loIL-7Rlo effector T cells were found in the RP and WP at an ~40:60 ratio, but PTX treatment blocked their migration into the WP (Fig. 2D). In contrast, KLRG1hiIL-7Rlo effector cells homed into the RP, regardless of the PTX treatment. These data suggest that the homing of KLRG1hiIL-7Rlo memory precursor effector cells to the T cell zone in the spleen is a chemokine receptor-dependent process.

Differential regulation of CCR7 expression in effector T cell subsets results in distinct homing abilities

Because the majority of the KLRG1loIL-7Rhi effector and memory CD8 T cells could migrate to the T cell zone, whereas the KLRG1hiIL-7Rlo cells could not, we examined the expression and function of several homing receptors to identify those that may be involved. A comparison of the gene-expression profiles of IL-7Rhi and IL-7Rlo LCMV-specific effector CD8+ T cells using Affymetrix microarrays showed that the mRNAs of several chemokine receptors and the sphingosine 1-phosphate receptor 5 were differentially expressed (Table I) (12). The chemokine receptors Ccr7, Ccr6, and Cxcr5 were elevated in IL-7Rlo effector CD8 T cells relative to IL-7Rhi effector cells. Conversely, the mRNAs for Cx3cr1 and Slpr5 were notably higher in IL-7Rhi effector than in IL-7Rlo effector cells. CCR7 is also required for T cell zone localization through recognition of the chemokines CCL19 and CCL21 produced by the gp38+ FRCs in the T cell zone in lymphoid organs (6, 43). CXC4 is important for extrafollicular migration of lymphocytes in the spleen and LNs, as well as migration to the bone marrow (BM), but its expression was not significantly different between the two subsets.

To further evaluate the gene expression and function of CCR7 and CXCR4 in the virus-specific CD8 T cells, we isolated effector and memory CD8 T cells from P14 chimeric mice infected with LCMV 9 and ~180 d prior and compared them with naive P14 CD8 T cells. Additionally, the day 9 effector cell population was separated into KLRG1hiIL-7Rlo terminal effector cells to KLRG1loIL-7Rhi effector cell subsets. As effector cells from the spleen were purified by FACS, based on their expression and function of CCR7 and CXCR4 using Affymetrix microarrays (Table I). CCR7 was expressed to a greater extent (14-fold) in KLRG1hiIL-7Rlo effector cells relative to KLRG1loIL-7Rhi memory precursor effector cells to compare these subsets. Quantitative RT-PCR showed that naive CD8 T cells expressed Ccr7 mRNA the most abundantly, but it was greatly repressed (70-fold) in day 9 effector D8 T cells (Fig. 3A). Consistent with the DNA microarray data (Table I), Ccr7 was expressed to a greater extent (14-fold) in KLRG1hiIL-7Rlo effector cells relative to KLRG1loIL-7Rhi. At ~6 mo p.i., the expression of CCR7 had increased considerably in the memory CD8 T cells, although the level continued to remain lower than that in naive CD8 T cells. Cxcr4 mRNA was also downregulated between naive and effector CD8 T cell stages, albeit to a lesser degree than Ccr7, but there was no significant difference between KLRG1hiIL-7Rlo and KLRG1hiIL-7Rhi effector cell subsets. As seen with Ccr7, Cxcr4 expression increased to a level similar to naive cells in memory CD8 T cells matured (Fig. 3B).

To investigate whether the differential expression of Ccr7 between KLRG1loIL-7Rhi and KLRG1hiIL-7Rhi CD8 T cells had functional consequences, we performed an in vitro Transwell-migration assay to compare the migratory capacities of the effector cell subsets to CCL19. In correlation with the mRNA expression, the migration of KLRG1loIL-7Rhi effector cells toward CCL19 was limited, whereas that of the KLRG1hiIL-7Rlo effector cells was considerably more efficient and occurred in a dose-dependent manner (Fig. 3C). In response to CXCL12, a ligand for CXCR4, both subsets were mobilized efficiently, in a dose-dependent fashion (Fig. 3D). Altogether, these data showed that KLRG1loIL-7Rhi and KLRG1hiIL-7Rhi effector T cells have distinct and overlapping chemokine responses (KLRG1loIL-7Rhi cells to CCL19 and CXCL12 and KLRG1hiIL-7Rlo effector cells to CXCL12), which likely contribute to the differential homing of these effector cells in lymphoid organs.
The above data suggest that CCR7 directs the localization of KLRG1loIL-7Rhi memory precursor effector cells to the T cell zone in the lymphoid organs. If so, then we suspected that these cells would preferentially be in close contact with gp38+ FRCs within the T cell zone that produce CCL21, CCL19, and IL-7. To examine this potential cell–cell interaction, the proximity of KLRG1loIL-7Rhi effector CD8 T cells to gp38+ CCL21+ FRCs was examined using immunofluorescent microscopy (Fig. 3E, 3F). This analysis showed that nearly all of the virus-specific CD8 T cells in the T cell zone were juxtaposed or in close association with the FRC stromal network. These results suggest an interesting model wherein T cell zones constitute an important survival niche for virus-specific memory CD8 T cells and their precursors. Among cells present in the T cell zone, FRCs may play the most important role in recruiting CCR7hiKLRG1lo effector cells by producing CCL21 and possibly providing extrinsic factors, such as IL-7, to these cells.

The transcription factors T-bet and Blimp-1 play crucial roles in effector T cell migration into the RP

We further investigated molecules that may be involved in regulating the differential migration patterns of effector T cell subsets. We were particularly interested in examining the transcription factors T-bet and Blimp-1, which were shown to modulate the expression of certain chemokine receptors and play a critical role in the development of terminally differentiated KLRG1hiIL-7Rlo effector CD8 T cells (12, 34, 35). In particular, gene expression-profiling experiments suggest that Blimp-1 represses Ccr7 and

Table I. Differential expression of chemokine receptors in LCMV-specific IL-7Rhi and IL-7Rlo effector cells

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>D8 IL-7Rhi versus IL-7Rlo</th>
</tr>
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<tbody>
<tr>
<td>CCR7</td>
<td>35.7</td>
</tr>
<tr>
<td>CCR6</td>
<td>8.3</td>
</tr>
<tr>
<td>CXCR5</td>
<td>5.5</td>
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<tr>
<td>CXCR6</td>
<td>1.8</td>
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<tr>
<td>CXCR4</td>
<td>1.1</td>
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<tr>
<td>CCR3</td>
<td>-1.8</td>
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<tr>
<td>CCR1</td>
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<tr>
<td>CCR4</td>
<td>-3.0</td>
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<tr>
<td>CX3CR1</td>
<td>-4.7</td>
</tr>
<tr>
<td>S1PR5</td>
<td>-20.5</td>
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</table>

Data are from three independent samples of LCMV-specific P14 IL-7Rhi and IL-7Rlo effector CD8 T cells sorted at day 7 p.i. and analyzed by Affymetrix U133a2 gene chips. Positive fold-change values are more highly expressed in IL-7Rhi cells; negative fold-change values are more highly expressed in IL-7Rlo cells. S1PR5, sphingosine 1-phosphate receptor 5.
Recent reports from our laboratory and others found that Sell (CD62L) expression, and T-bet and Blimp-1 promote the expression of Cx3cr1 and Slpr5 (35, 44) (N.S. Joshi and S.M. Kaech, unpublished observations).

To determine whether T-bet plays a role in effector T cell homing in the spleen, we used a retroviral system to overexpress T-bet. P14 CD8 T cells were activated with gp33 peptide in vitro and then transferred into naive mice i.v., and their location in the spleen was analyzed 5 d later by fluorescent microscopy of GFP cells. Frozen serial sections of the spleen from day 30 p.i. were stained with Ly5.1, KLRG1, and either CCL21 (E) or gp38 (F). Data are representative of six mice; multiple frozen sections per mouse were analyzed.

FIGURE 3. Differential expression and function of CCR7 in virus-specific effector CD8 T cell subsets. Quantitative RT-PCR analysis of CCR7 (A) and CXCR4 (B) mRNA expression in naive P14 cells, KLRG1hiIL-7Rlo cells, and KLRG1hiIL-7Rlo cells from day 9 p.i. and memory CD8+ T cells from day 180 p.i. Bar graphs show the relative amount of mRNA (mean ± SEM), compared with naive cells, which is representative of two independent experiments with three samples each. In vitro Transwell-migration assay for CCL19 (C) and CXCL12 (D). Effector CD8 T cells from day 9 p.i. migrated in Transwell toward various concentration of CCL19 or CXCL12 for 3 h were analyzed by flow cytometry. Migrating cells were counted based on surface markers and compared with the number of cells originally dispersed. The background was subtracted. E and F. Memory T cells in the T cell zone appeared to make contact with FRCs. Half of the spleens were used to determine the phenotype of the remaining spleen portions were used to examine the phenotype of P14 subsets by flow cytometry. As expected (35), the majority of P14 Prdm1 KO cells exhibited a KLRG1hiIL-7Rlo phenotype (Fig. 5A). The remaining spleen portions were used to identify the localization of the Prdm1 WT and KO P14 cells by immunofluorescent microscopy. Compared with WT control cells, P14 cells lacking Prdm1 expression were predominantly found in the T cell zone and exhibited a KLRG1hiIL-7Rlo phenotype (Fig. 5A). Over-all, these data strongly indicate that T-bet and Blimp-1 play key roles in driving activated CD8 T cells out of the T cell zone and that effector CD8 T cells that express lower levels of these factors can express CCR7 and migrate into the T cell zone.

Tcells localize to the T cell zone, whereas Tcells are mostly found in the RP.

Next, we assessed whether TCM and TEM cells, which differ in CD62L and CCR7 expression, also localize to different areas of the spleen using immunofluorescent microscopy. At day 60 p.i., most of the splenic memory CD8 T cells are IL-7Rlo, and ~30% of these cells are CD62Llo TEM cells, whereas ~70% are CD62Lhi TCM cells. A small proportion (20%) of IL-7RloCD62Llo TEM cells are also present at this time point (Fig. 6A). Consistent with Fig. 1,
P14 Ag-specific memory CD8 T cells were dispersed in the T cell zone, B cell zone and the RP; however, CD62L<sup>hi</sup> memory CD8 T cells were predominantly found in the T cell zone (Fig. 6B, 6C). In contrast, most of the memory CD8 T cells found in the RP were CD62L<sup>lo</sup> T<sub>EM</sub> cells.

Given the distinct locations of T<sub>CM</sub> and T<sub>EM</sub> cells in the WP and RP of the spleen, respectively, we tested the ability of the three memory CD8 T cell subsets to migrate toward CCL19 and CXCL12 in Transwell-migration assays. IL-7R<sup>hi</sup>CD62L<sup>lo</sup> memory cells did not respond to CCL19; however, as expected, IL-7R<sup>hi</sup> memory cells migrated toward this chemokine. Among IL-7R<sup>hi</sup> memory T cells, CD62L<sup>+</sup> T<sub>CM</sub> cells migrated better toward CCL19 than did CD62L<sup>−</sup> T<sub>EM</sub> cells (Fig. 6D). On the contrary, IL-7R<sup>lo</sup>CD62L<sup>lo</sup> T<sub>EM</sub> cells were more mobile than were IL-7R<sup>lo</sup>CD62L<sup>hi</sup> T<sub>CM</sub> cells and IL-7R<sup>lo</sup>CD62L<sup>lo</sup> memory cells in response to CXCL12 (Fig. 6E). Altogether, these data show that T<sub>CM</sub> and T<sub>EM</sub> cells (based on CD62L expression) have distinct homing preferences in the spleen, and this directly correlates with their differential responses to CCR7 and CXCR4 ligands, respectively. Thus, in support of a recent report, one would predict their differential responses to CCR7 and CXCR4 ligands, re-homing preferences in the spleen, and this directly correlates with their survival, maintenance, and ability to protect against secondary infection.

The localization of splenic effector and memory CD8 T cells during infection was described previously. The first report showed that most effector T cells migrated into the RP, and this correlated with repression of Ccr7 mRNA expression (45). Another group used in situ staining of endogenous effector T cells after L. monocytogenes infection (46, 47). Using an MHC class I tetramer-staining technique, that study showed that naive T cells were activated in the T cell zone by APCs and that activated effector T cells migrated from the T cell zone to the RP. Interestingly, after this type of infection, memory T cells were mostly found in the B cell zone, marginal zone, and the RP. Another study used a Granzyme B-cre ROSA-YFP reporter mouse strain that marks virus-specific effector CD8 T cells indefinitely with YFP<sup>+</sup> (47). Using these mice, the localization of effector T cells was examined based on YFP expression. In agreement with these studies, we found that most effector cells were localized in the RP, however, a small, but sizable, number of T cells was also found in the T cell zone. Our further analysis demonstrated that this small population consisted of KLRG1<sup>hi</sup>IL-7R<sup>lo</sup> memory precursor effector cells, whereas the majority of effector T cells on day 8 p.i. were KLRG1<sup>lo</sup>IL-7R<sup>lo</sup> and were found in the RP. These data provide an important extension from these prior studies, because they show that the homing patterns of the antiviral CD8 T cells are not random in tissues but are largely influenced by their differentiation states.

We found that KLRG1<sup>hi</sup>IL-7R<sup>lo</sup> effector T cells homed preferentially to the T cell zone, presumably as the result of greater expression of CCR7 relative to KLRG1<sup>lo</sup>IL-7R<sup>lo</sup> cells. Interestingly, the expression of Ccr7 mRNA increased over time as memory CD8 T cells matured. How is CCR7 expression differentially regulated on the effector CD8 T cell subsets? Unlike CD62L<sup>+</sup>, it was shown that CCR7 is not downregulated in CD8 T cells directly after peptide stimulation in vitro (45), indicating that TCR signaling is not sufficient to repress CCR7 on activated T cells. It is likely that the inflammatory cytokines to which cells are exposed during priming and infection are involved. We previously reported that IL-12 is a potent inducer of T-bet expression in CD8 T cells and augments the development of terminal KLRG1<sup>hi</sup>IL-7R<sup>lo</sup> effector cells (12). Given that overexpression of T-bet was sufficient to expel activated CD8 T cells out of the WP and into the RP, we hypothesize that T-bet might suppress Ccr7 expression and upregulate chemokine receptors that induce

**Discussion**

The development and maintenance of long-lived functional memory T cells are critical for protective immunity. This study focused on how environmental factors impact effector and memory CD8 T cell development and maintenance by analyzing their localization during infection. First, we noted that subsets of effector T cells differed in their expression of chemokine receptors, especially CCR7. KLRG1<sup>hi</sup>IL-7R<sup>lo</sup> memory precursors expressed a higher level of CCR7 mRNA and showed greater migratory capacity toward CCL19 compared with KLRG1<sup>lo</sup>IL-7R<sup>lo</sup> terminal effector cells. Although KLRG1<sup>lo</sup>IL-7R<sup>lo</sup> effector cells were predominantly localized in the RP of the spleen, KLRG1<sup>lo</sup>IL-7R<sup>lo</sup> T cells actively homed into the T cell zone and the RP. Overexpression of T-bet induced migration of T cells into the RP, whereas KLRG1<sup>lo</sup>IL-7R<sup>lo</sup> effector T cells deficient in Prdm1 expression predominantly localized to the T cell zone. In addition, T<sub>CM</sub> and T<sub>EM</sub> cells localized in different microenvironments of the spleen. Altogether, these data show that the anatomical location of effector and memory CD8 T cells can be regulated by their differentiation state in a transcription-dependent manner, which may directly affect their survival, maintenance, and ability to protect against secondary infection.

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migration into the periphery (48). Likewise, IL-2 can induce Blimp-1 in activated CD8 T cells, and IL-2 and Blimp-1 promote the development of terminal KLRG1<sup>hi</sup>IL-7R<sup>lo</sup> effector cells (30, 31, 34, 35, 49). In vitro culture with high-dose IL-2 can generate TEM-like CD8 T cells via PI3K/Akt-dependent signaling events that repress Ccr7 and Sell (CD62L) expression (50, 51). Moreover, Blimp-1 is likely a direct repressor of Ccr7 and Sell expression because these loci contain multiple putative Blimp-1–binding sites, and their expression is increased considerably in LCMV-specific Prdm1<sup>−/−</sup> effector T cells relative to WT cells (35). Thus, differential exposure to IL-2 and Blimp-1 activity also regulates the migration of virus-specific effector and memory CD8 T cells.

It is possible that the increased CCR7 expression and T cell zone homing contribute to the longevity and recall capability of the KLRG1<sup>hi</sup>IL-7R<sup>lo</sup> effector and memory T cells because this enables them to stay in contact with the gp38<sup>+</sup> FRCs that secrete IL-7 and CCL19/21 (6). In support of this, our data above showed that Prdm1 KO effector cells have increased Ccr7 expression and accumulate more densely in the T cell zone compared with WT cells. Interestingly, this property corresponds well with the enhanced longevity observed in Prdm1 KO memory CD8 T cells relative to WT cells. Lastly, the inability to home to the T cell zone may be associated with reduced memory CD8 T cell numbers after LCMV infection (52).

The inability of KLRG1<sup>hi</sup> CD8 T cells to home to the T cell zone may contribute to their shortened life span. Our previous study showed that overexpression of IL-7R on virus-specific CD8 T cells could not prevent the death of the KLRG1<sup>hi</sup> terminal effector cells. One possibility is that most KLRG1<sup>hi</sup> effector cells are programmed to die, and this was unaffected by increased IL-7R signaling. Alternatively, in light of our data, it may be that the IL-7R–overexpressing KLRG1<sup>hi</sup> cells were unable to “see” IL-7 because they were effectively mislocalized away from IL-7 niches. Although soluble IL-7 can be detected in the serum, it likely remains closely tethered to the extracellular matrix where it is produced; this may create niches, similar to cryptopatches in the intestine, which support the survival of IL-7R<sup>hi</sup> T cells (53, 54). The liver and BM are likely other relevant sources of IL-7 for memory CD8 T cells (3, 55, 56), and it will be important to better understand the cell types that produce IL-7 in these tissues, too. Additional benefits from T cell zone homing may be increased contact with Ag-presenting DCs that would permit faster and more protective responses to secondary infection that was demonstrated by KLRG1<sup>lo</sup>IL-7R<sup>hi</sup> CD8 T cells relative to KLRG1<sup>hi</sup>IL-7R<sup>lo</sup> cells.

In contrast, optimal control of infections in peripheral tissues requires that Ag-specific CD8 T cells migrate out of the T cell zones and into the periphery (43, 48, 57). For instance, LCMV-specific CD8 T cells overexpressing CCR7 were largely trapped in the lymphoid organs, and this led to reduced protection against secondary infection at peripheral sites (58). Following LCMV, Listeria, and influenza infection, KLRG1<sup>hi</sup> effector CD8 T cells dominate in the nonlymphoid peripheral tissues (12, 34). These data support a model wherein a division of labor exists among
effector T cells (59); those that migrate into the periphery to combat infection are more likely to differentiate into terminal effector cells that lose longevity and memory cell potential, whereas those that remain in the T cell zone are biased to develop into long-lived memory precursor cells. Although this model is tempting because it simplifies the creation of different effector cell fates, it is evident that there is no strict tissue compartmentalization of IL-7Rhi memory CD8 T cells and their precursors, because IL-7Rhi Ag-specific CD8 T cells have been observed in many peripheral tissues, including lung and liver. Thus, it is likely that there are several IL-7–dependent niches throughout the body.

Lastly, because the survival of KLRG1hi effector and memory CD8 T cells is acutely IL-15 dependent (12, 60), it is likely that interactions with IL-15–presenting cells occurs in the RP of the spleen and in other tissues where KLRG1hi CD8 T cells predominate. Based on a recent study, macrophages are the most likely IL-15–presenting cell type to sustain KLRG1hi TEM cells, although another study showed that DCs can also suffice (8, 11). In contrast, IL-15–presenting DCs are selectively required for TCM cell homeostasis (8). In conjunction with our data, we predict that these cells largely reside in the T cell zone. Moreover, IL-15 can induce the expression of the costimulatory molecule 4-1BB on memory T cells, and 4-1BB:4-1BBL interactions are crucial for the survival of virus-specific memory CD8 T cells (61). It will be interesting to see whether IL-15–presenting cells and 4-1BBL+ cells are the same and where they are localized in tissues.

The work presented in this article shows that the differentiation state of virus-specific effector and memory CD8 T cells can greatly influence their chemokine receptor-expression patterns and homing to distinct tissues, as well as to distinct microenvironments within tissues. This, in turn, results in different types of cell–cell interactions between the T cells and other cells in these areas that likely affect their survival, turnover, and function. Therefore, this finding provides an important advancement toward a better understanding of the homeostasis and regulation of the numerous effector and memory T cell populations. Further examination of the cell types and signals provided in these specialized microenvironments will help us to better understand how memory T cells develop and persist in vivo following infections, and this could lead to new types of vaccines and treatments that may enhance protection against infectious disease and cancer.

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Disclosures

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