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Strength of Stimulus and Clonal Competition Impact the Rate of Memory CD8 T Cell Differentiation1,2

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The developmental pathways of long-lived memory CD8 T cells and the lineage relationship between memory T cell subsets remain controversial. Although some studies indicate the two major memory T cell subsets, central memory T (T CM) and effector memory T (T EM), are related lineages, others suggest that these subsets arise and are maintained independently of one another. In this study, we have investigated this issue and examined the differentiation of memory CD8 T cell subsets by tracking the lineage relationships of both endogenous and TCR transgenic CD8 T cell responses after acute infection. Our data indicate that TCR transgenic as well as nontransgenic T EM differentiate into T CM in the absence of Ag. Moreover, the rate of memory CD8 T cell differentiation from T EM into the self-renewing and long-lived pool of T CM is influenced by signals received during priming, including Ag levels, clonal competition, and/or the duration of infection. Although some T EM appear not to progress to T CM, the vast majority of T CM are derived from T EM. Thus, long-lasting, Ag-independent CD8 T cell memory results from progressive differentiation of memory CD8 T cells, and the rate of memory T cell differentiation is governed by events occurring early during T cell priming. The Journal of Immunology, 2007, 179: 6704–6714.

Memory CD8 T cells possess several defining characteristics that distinguish them from their naive and effector T cell counterparts. First, memory CD8 T cells can persist for extended periods of time in the absence of Ag (1–3). Second, memory CD8 T cells undergo slow, steady homeostatic proliferation driven by IL-7 and IL-15 (1, 4, 5). This self-renewing division is essential for long-term maintenance of the memory T cell population (5, 6). Neither naive nor effector CD8 T cells are capable of long-term self-renewal and persistence via IL-7- and IL-15-driven homeostatic proliferation under normal conditions. These properties are acquired by memory CD8 T cells during their development following the effector phase (7). In addition, memory T cells have the ability to rapidly reactivate effector functions and undergo vigorous proliferation in response to Ag stimulation, and these two properties are central to the ability of memory T cells to confer robust protective immunity (3, 7–9). Our understanding of the lineage development of memory CD8 T cells and the signals that govern the generation of persisting and protective memory CD8 T cells, however, remains incomplete.

Memory T cells can be divided into several subsets based on phenotype, function, and/or anatomical location. For example, effector memory T (T EM)7 are CD62Llow and CCR7low, do not efficiently produce IL-2, home inefficiently to lymph nodes (LN), and are often found in nonlymphoid tissues (10, 11). In contrast, central memory T (T CM) are CD62Lhigh and CCR7high, can produce IL-2 upon stimulation, and can efficiently enter LN (10). It is also the T CM subset that undergoes efficient homeostatic proliferation and self-renewal (9). Although several studies have addressed the lineage relationship between T EM and T CM, this topic remains controversial. In vitro studies found that T EM-like and T CM-like CD8 T cells may be generated under different conditions, suggesting independent lineages (12). TCR sequencing, however, indicates shared TCR clonotypes between T EM and T CM subsets, suggesting that many T EM and T CM have a common origin (13, 14). Our previous work using TCR transgenic approaches demonstrated that within the memory population T EM and T CM are related lineages and that T EM can gradually convert to T CM over time in the absence of Ag (9). This T EM to T CM conversion is also accompanied by acquisition of several key memory T cell properties, including phenotypic conversion from CD62Llow to CD62Lhigh (as well as CCR7low to CCR7high and CD27low to CD27high), acquisition of the ability to produce IL-2, robust proliferative potential, and the ability to undergo self-renewing homeostatic proliferation (9). More recently, Marzo et al. (15) reported that whereas TCR transgenic CD8 T cells converted from T EM to T CM within the memory pool, a similar pattern of memory CD8 T cell differentiation was not observed when the Ag-specific T cell precursor frequency was low, as is the case for non-TCR transgenic endogenous virus-specific CD8 T cell populations. It

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7 Abbreviations used in this paper: T EM, effector memory T; LCMV, lymphocytic choriomeningitis virus; LCMV Arm, LCMV Armstrong; LN, lymph node; NP, nuclear protein; p.i., postinfection; T CM, central memory T; VSV, vesicular stomatitis virus; VSV-Indiana.

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was proposed that the lineage relationships and differentiation of memory CD8 T cell subsets were different for TCR transgenic models with high precursor frequency compared with natural, endogenous T cell responses (15). Thus, the precise pattern of memory T cell differentiation resulting in the generation of self-renewing TCM remains to be completely defined. Moreover, the signals that govern the pattern of memory T cell development and the dynamic changes in the proportions of TEM and TCM within the memory T cell population remain poorly understood.

In this study, we have addressed these issues by examining the lineage and differentiation of memory CD8 T cell subsets using both TCR transgenic approaches and by examining the endogenous nontransgenic response to viral infection. Our results demonstrate that, like TCR transgenic memory CD8 T cells, endogenous nontransgenic virus-specific memory CD8 T cell populations responding to multiple epitopes undergo TEM to TCM conversion over time in the absence of Ag. Importantly, the rate of this TEM to TCM conversion depends upon the strength and/or duration of the stimulus and occurs more slowly for the endogenous virus-specific CD8 T cell responses compared with TCR transgenic approaches. Endogenous CD8 T cells specific for strongly presented epitopes undergo TEM to TCM conversion more slowly than those populations responding to subdominant epitopes, indicating that the level of Ag stimulation may impact the rate of TEM to TCM conversion in the memory CD8 T cell pool. Purified endogenous TEM were capable of directly converting into TCM over time, and this conversion was associated with enhanced homeostatic proliferation.

The rate of memory T cell differentiation for the endogenous virus-specific CD8 T cell responses is slower than typically observed for TCR transgenic models, suggesting a role for T cell competition in regulating the rate of memory T cell differentiation. Indeed, when lower numbers of TCR transgenic T cells were used (i.e., less competition), the rate of memory T cell differentiation was more protracted. If more naive TCR transgenic T cells were present, or if Ag-specific CD8 T cells are exposed to a shortened duration of infection, the rate of memory T cell differentiation became more rapid. Thus, these results demonstrate that the development of self-renewing memory CD8 T cells is the result of a linear differentiation of TEM into TCM for both endogenous and TCR transgenic T cells. Moreover, our data demonstrate that the rate of this memory CD8 T cell differentiation is influenced by the level of Ag stimulation, clonal competition, and/or the strength of initial stimulation.

Materials and Methods

Mice, virus, and infections

C57BL/6/J (Thy1.2) and C57BL/6J (Ly5.1) mice were purchased from The Jackson Laboratory. Thy1.1+/P6.1 mice bearing the Db-gp33-specific TCR were fully backcrossed to C57BL/6 and maintained in our animal colony. Lymphocytic choriomeningitis virus (LCMV) Armstrong (LCMV Arm) and vesicular stomatitis virus (VSV) Indiana (VSV-Indiana) viruses were propagated, titrated, and used, as described (9, 16). B6 mice were infected with LCMV Arm with 2 × 10^4 PFU i.p. or with 1 × 10^4 PFU. VSV-Indiana i.v. P14 chimeric mice were generated by adoptively transferring 10^5, 10^4, or 10^3 naive TCR transgenic T cells into naive B6 mice, followed by LCMV Arm infection (referred to as P14 chimeras). All mice were used in accordance with National Institutes of Health and the Emory University and Wistar Institute Institutional Animal Care and Use Committee guidelines.

Isolation of T cells, purification of CD62L^high and CD62L^low CD8 T cells, and CFSE labeling

Lymphocyte populations were purified using anti-CD62L or anti-CD8 magnetic beads (Miltenyi Biotec), according to the manufacturer’s instructions or by flow cytometric sorting. The purity of FACS-sorted samples was ~99%, and purity of cells isolated by magnetic beads ranged from 95 to 99%. Cells were labeled with CFSE for homeostatic proliferation analyses (Molecular Probes), as described previously (9). BrdU incorporation was examined by administering 0.8 mg/ml in drinking water for 1 wk, following which BrdU incorporation in CD8 T cells was measured by staining for intracellular BrdU (BD Biosciences), according to the manufacturer’s instructions. Lymphocytes were isolated from nonlymphoid tissues, as described (9).

Flow cytometry and intracellular cytokine staining

MHC class I peptide tetramers were made and used as described. All Abs were purchased from BD Biosciences, except Ab to mouse IL-7R (anti-mouse IL-7R) from eBioscience and anti-human granelyme B from Caltag Laboratories. Cells were stained for surface proteins or intracellular proteins and cytokines, as described (9). For analysis of intracellular cytokines, lymphocytes were stimulated with LCMV peptides (0.2 μg/ml) in the presence of brefeldin A for 5 h, followed by surface staining for CD8 and intracellular staining for IFN-γ, TNF-α, or IL-2.

Results

Endogenous LCMV-specific memory CD8 T cells gradually differentiate from TEM to TCM.

To examine the kinetics of memory CD8 T cell differentiation for non-TCR transgenic epitope-specific memory CD8 T cell populations, C57BL/6 mice were infected with LCMV Arm and LCMV-specific CD8 T cell populations were examined longitudinally in the blood. Three different LCMV-specific CD8 T cell populations were identified by staining with MHC tetramers for one LCMV nuclear protein (NP) (NP396) and two glycoprotein (gp33 and gp276) epitopes. LCMV NP protein is produced earlier and at a higher level during infection than glycoprotein (17–19), and this pattern of protein expression corresponds well to the higher level of NP396 epitope available for T cell stimulation compared with the glycoprotein epitopes (20). The expression of CD62L by the Db/NP396, Db/gp33, and Db/gp276 tetramer^+ CD8 T cell populations was determined over time. As shown in Fig. 1A, there was a distinct kinetics for the change CD62L expression associated with each epitope-specific CD8 T cell population. For example, the CD62L^low to CD62L^high conversion of the Db/NP396-specific CD8 T cell population occurred more slowly compared with the Db/gp33- and Db/gp276-specific populations. The number of days until the memory CD8 T cell population was 50% CD62L^high (T_{50\%}) provides a useful comparison of the rate of change of CD62L expression in these memory CD8 T cell populations (Fig. 1B). Indeed, whereas it took >150 days for the Db/NP396-specific CD8 T cell population to reach 50% CD62L^high, the Db/gp33- and Db/gp276-specific populations reached this point by ~100 days postinfection (p.i.). Similar population conversion from CD62L^low to CD62L^high also occurred for the non-TCR transgenic CD8 T cell response to VSV (T_{50\%} ~150 days; Fig. 1D), suggesting that the pattern of CD62L expression presented in Fig. 1, A and B, may be a common feature of the endogenous CD8 T cell response to multiple viral infections. It should be pointed out that this analysis, and the studies presented below, are for Ag-specific memory CD8 T cells following a primary infection in the absence of re-exposure to Ag.

Expression of CD62L is one marker that distinguishes TCM from TEM. To examine a functional correlate of memory CD8 T cell differentiation, we next examined IL-2 production by the same three populations of endogenous LCMV-specific memory CD8 T cells at different times p.i. IL-2 production gradually increases over time as the memory CD8 T cell population differentiates from TEM into TCM (9, 21). As shown in Fig. 1C, the rate at which these memory CD8 T cell populations acquire the ability to produce IL-2 upon peptide stimulation also differed. The gp276-specific population acquired the ability to produce IL-2 most rapidly, followed by gp33 and then by the NP396-specific population, which acquired this property more slowly.
Two important points can be made from these data. First, two different aspects of memory T cell differentiation, conversion of the population from CD62Llow to CD62Lhigh and the ability to produce IL-2, change over time at rates that differed for each epitope-specific memory CD8 T cell population. Second, these data suggest that the strength of the antigenic stimulus influences the rate of memory CD8 T cell differentiation because all three epitope-specific CD8 T cell populations responding to acute LCMV infection are stimulated in the same inflammatory environment, yet the memory CD8 T cell population responding to the LCMV Db/gp33 epitope differed in different tissues.

Endogenous LCMV-specific memory CD8 T cell differentiation also occurred in multiple tissues

It was possible that the data in Fig. 1 simply reflected redistribution of memory CD8 T cells out of the blood and spleen and/or did not accurately reflect the situation in different tissues. To address this issue, CD62L expression by LCMV Db/gp33-specific CD8 T cells was examined in the spleen, LN, bone marrow, and liver at multiple time points p.i. As shown in Fig. 2, A and B, in all tissues examined, including the liver, the population of Db/gp33-specific CD8 T cells gradually changed from CD62Llow to CD62Lhigh over time p.i. The rate of CD62Llow to CD62Lhigh conversion for Db/gp33-specific memory CD8 T cells differed in different tissues. The memory CD8 T cells in the spleen and blood had very similar rates of CD62Llow to CD62Lhigh change, and the reversion in the bone marrow was only marginally more rapid. The similarity in reversion rates for these tissues may indicate that the memory CD8 T cells in these locations are part of a systemic recirculating pool of memory T cells, as has been suggested by parabiosis experiments (22). In contrast, conversion in the LN occurred more rapidly, whereas the memory CD8 T cells in the liver had a more protracted course of CD62Llow to CD62Lhigh conversion. The change in memory CD8 T cells in the LN most likely reflected migratory preferences because only CD62Lhigh memory CD8 T cells will have access to this location. It is also possible that the slow rate of reversion in the liver reflected migratory preferences or, alternatively, the difference in reversion in this location could reflect a liver-resident population of memory CD8 T cells that differentiated more slowly in situ. It should be mentioned that in some locations, such as the intraepithelial compartment of the gut and in the brain, reversion to CD62Lhigh was not observed even after prolonged periods of time (23) (data not shown). The phenotypic changes observed in the tissues were also accompanied by functional changes. IL-2 production by spleen-derived memory CD8 T cells occurred more rapidly compared with acquisition of this property by the memory T cell population derived from the liver (Fig. 2B). Finally, these changes in memory CD8 T cell populations in the
tissues occurred under conditions in which the total number of Dbgp33-specific CD8 T cells remained relatively constant over time (Fig. 2D). This latter observation suggests that it is unlikely that one subpopulation of memory CD8 T cells is simply outcompeting or outproliferating the other. Rather, these observations are more consistent with one subpopulation of memory T cells converting into another.

**Clonal competition between T cells alters the rate of memory CD8 T cell differentiation**

TCR transgenic T cells have been suggested to undergo a different program of memory CD8 T cell differentiation compared with endogenous memory CD8 T cells (15). To further investigate this issue, we next compared the rate of CD62L re-expression by a population of Db/gp33-specific TCR transgenic CD8 T cells (P14 cells) with the population of endogenous Db/gp33-specific CD8 T cells. When compared directly in separate mice, the TCR transgenic memory CD8 T cell population converted from CD62Llow to CD62Lhigh more rapidly than the nontransgenic memory CD8 T cell population (Fig. 3A; T_{50%} = 68 vs 99 days). Next, P14 chimeras were made using different numbers of initial TCR transgenic T cells. High (10^6), intermediate (10^5), and low (10^3) doses of P14 cells were adoptively transferred to naive mice before LCMV Arm infection. The lowest dose of 10^3 donor P14 cells approximates the number of endogenous Db/gp33-specific memory CD8 T cells in C57BL/6 mice (24). As shown in Fig. 3, B and C, the initial dose of P14 cells impacts both the total number of P14 cells recovered and the timing and magnitude of the peak expansion following LCMV Arm infection. At the lowest dose of P14 cells used, both the timing and magnitude of the P14 expansion most closely reflected the endogenous Db/gp33 response (see Fig. 2D and Refs. 20, 21, 24, and 25). The initial precursor frequency of P14 CD8 T cells also impacts the intensity of effector CD8 T cell differentiation. Early in the effector T cell response, at day 4 p.i., granzyme B expression levels were similar between the three doses of P14 cells transferred (Fig. 3D). By day 6 p.i., however, at the two higher doses of P14 cells transferred, the expression level of granzyme B was substantially lower compared with when the only 10^3 P14 cells were transferred (compare mean fluorescence intensity of 122 and 119 vs 221 on day 6; Fig. 3D). This observation suggests that when clonal competition is low, Ag-specific CD8 T cells receive stronger or more prolonged stimulation. The P14 precursor frequency also impacts the rate at which the P14 cells acquire a CD127^{high} phenotype (Fig. 3E). When the precursor frequency is high (e.g., 10^6), the developing memory CD8 T cell pool becomes CD127^{high} more rapidly compared with when a low number of P14 CD8 T cells was used.

**FIGURE 2.** Kinetics of CD62L expression and IL-2 production by endogenous LCMV-specific CD8 T cells in different tissues. A, CD62L expression was examined on Dbgp33 tetramer^+ endogenous CD8 T cells from the spleen, LN, bone marrow, and liver at the indicated time points following LCMV Arm infection. All histograms are gated on CD8^+ Dbgp33^+ T cells. B, Summary of CD62L expression on Dbgp33-specific CD8 T cells in the tissues shown in A. C, IL-2 production by gp33-specific CD8 T cells in spleen and liver of LCMV-immune mice at the indicated time points.

Summary of CD62L expression on Dbgp33-specific CD8 T cells in the tissues shown in Fig. 2. The initial dose of P14 cells impacts both the total number of P14 cells recovered and the timing and magnitude of the peak expansion following LCMV Arm infection. At the lowest dose of P14 cells used, both the timing and magnitude of the P14 expansion most closely reflected the endogenous Db/gp33 response (see Fig. 2D and Refs. 20, 21, 24, and 25). The initial precursor frequency of P14 CD8 T cells also impacts the intensity of effector CD8 T cell differentiation. Early in the effector T cell response, at day 4 p.i., granzyme B expression levels were similar between the three doses of P14 cells transferred (Fig. 3D). By day 6 p.i., however, at the two higher doses of P14 cells transferred, the expression level of granzyme B was substantially lower compared with when the only 10^3 P14 cells were transferred (compare mean fluorescence intensity of 122 and 119 vs 221 on day 6; Fig. 3D). This observation suggests that when clonal competition is low, Ag-specific CD8 T cells receive stronger or more prolonged stimulation. The P14 precursor frequency also impacts the rate at which the P14 cells acquire a CD127^{high} phenotype (Fig. 3E). When the precursor frequency is high (e.g., 10^6), the developing memory CD8 T cell pool becomes CD127^{high} more rapidly compared with when a low number of P14 CD8 T cells was used.
FIGURE 3. Clonal competition influences the rate of memory CD8 T cell differentiation. A, Rate of CD62L expression by Dbgp33-specific TCR transgenic (P14) CD8 T cells compared with endogenous Dbgp33-specific CD8 T cells following LCMV Arm infection of separate unmanipulated C57BL/6 mice and P14 chimeras (10^6 P14 cells transferred). Dashed lines indicate T_{50%} for CD62L. B, Longitudinal analysis of Dbgp33-specific TCR transgenic P14 cells in the PBMCs after LCMV Arm infection of C57BL/6 mice that received 10^6 (high), 10^5 (intermediate), or 10^3 (low) P14 cells 1 day before infection. C, Total numbers of P14 cells recovered from the spleens of mice from B (left panel). Fold expansion of P14 transgenic CD8 T cells was determined as the ratio of absolute cell numbers at their respective peaks of expansion and initial precursor frequency (take estimated at ~10% of transferred cells in all cases). Peak number and fold expansion were determined at days 6, 7, and 8 P.I. for 10^6, 10^5, and 10^3 donor P14 cells, respectively. Graphs are representative of three independent experiments and n = 5 – 6. D, Intracellular granzyme B expression at high, intermediate, or low doses of P14 CD8 T cells. All histograms are gated on CD8^+ Thy1.1^+ P14 cells, and numbers in red indicate the mean fluorescence intensity (MFI) of granzyme B expression. E, Time post infection and CD8^+ P14 cells transferred. F, Comparison of CD62L expression on endogenous Dbgp33-specific and donor P14 Dbgp33-specific CD8 cells at indicated times P.I. for the 10^6 dose of P14 cells. Note that at this dose of P14 cells, ~50% of the Dbgp33 tetramer^+ population is contributed from host and 50% from the donor P14 cells at all time points examined. Histograms are gated on host (left) Thy1.1^+ CD8^+ Dbgp33^+ or donor P14 (right) Thy1.1^+ CD8^+ Dbgp33^+ cells, and the expression of CD62L for each population is shown.

The data presented in Fig. 3, A–E, suggest that the precursor frequency of P14 CD8 T cells may impact the rate of memory CD8 T cell differentiation of the adoptively transferred P14 cells. We next compared the memory CD8 T cell differentiation of the donor P14 TCR transgenic CD8 T cells with the Db/gp33-specific non-transgenic endogenous response present in the same mice when the initial precursor frequency of P14 cells (10^3 transferred) approximated the endogenous naive Db/gp33-specific population. There was an equal contribution from host and donor CD8^+ T cells in the Db/gp33 tetramer^+ population, and this ratio remained constant for at least 125 days (Fig. 3F). In addition, the memory CD8 T cell differentiation profile of host and donor Db/gp33-specific CD8 T cells was essentially indistinguishable, with both populations acquiring a CD62L^high phenotype with very similar kinetics (Fig. 3F).

Together, these observations suggest that the rate of memory CD8 T cell differentiation was influenced by clonal competition. When clonal competition was high, the rate of memory CD8 T cell differentiation was rapid; when competition was low, the rate of this memory T cell differentiation process was more protracted. When present at similar precursor frequencies, and thus, exposed to a similar antigenic and inflammatory environment, TCR transgenic P14 cells and host non-TCR transgenic virus-specific CD8 T cells undergo a similar pattern of memory CD8 T cell differentiation. Thus, TCR transgenic and endogenous T cells do not appear to undergo inherently different patterns of memory T cell differentiation. Rather, the signals received during priming, including the level of Ag, the inflammatory environment, and/or clonal competition most likely shape the kinetics of TEM to TCM conversion in the memory pool.

The duration of stimulation influences the rate of memory T cell CD62L^low to CD62L^high conversion

We next investigated how altering the duration of stimulus would impact memory CD8 T cell differentiation using a different approach. For these experiments, naive P14 CD8 T cells were adoptively transferred to LCMV Arm-infected mice on day 0 or at 4 days p.i. Because LCMV Arm infection is cleared from adult C57BL/6 mice by day 8–9 p.i. (20, 26), this experimental approach results in one group of P14 CD8 T cells (day 0 transfer) exposed to ~8–9 days of infection and stimulation, and a second population (day 4 transfer) only exposed to 4–5 days of stimulation. The expression of CD62L as a marker of memory CD8 T cell
Endogenous memory CD8 T cells can undergo direct conversion from CD62L<sub>low</sub> to CD62L<sub>high</sub>

One model of memory CD8 T cell differentiation suggests that whereas TCR transgenic TEM can convert to TCM, during the response of endogenous antiviral CD8 T cells the TEM and TCM subsets are separate lineages and TEM do not convert to TCM (15). Given our present observations indicating that the rate of CD62L<sub>low</sub> to CD62L<sub>high</sub> change in the endogenous virus-specific CD8 T cell population may be slower than for TCR transgenic T cells, we next re-examined whether endogenous LCMV-specific CD8 T cells could undergo direct TEM to TCM differentiation. To test this possibility, C57BL/6 mice were infected with LCMV Arm. Three months p.i., CD62L<sub>high</sub> and CD62L<sub>low</sub> memory CD8 T cells were purified, CFSE labeled, and adoptively transferred to new mice. Fig. 5A demonstrates that the CD62L<sub>high</sub> donor Db/ NP396-specific memory CD8 T cells remained CD62L<sub>high</sub> over the course of 85 days posttransfer (~175 days p.i.). In contrast, the CD62L<sub>low</sub> donor Db/NP396-specific CD8 T cell subset gradually converted from CD62L<sub>low</sub> to CD62L<sub>high</sub>, and at least some of this conversion occurred before homeostatic division. Importantly, the rate of this CD62L<sub>low</sub> to CD62L<sub>high</sub> conversion was consistent with the rate of CD62L<sub>low</sub> to CD62L<sub>high</sub> conversion observed for the total Db/NP396-specific CD8 T cell population (120 days in Fig. 5B compared with 152 days in Fig. 1). Similar results were observed for the endogenous Db/gp33-specific CD8 T cell population (Fig. 5C). It is also interesting to point out that the rate of CD62L<sub>low</sub> to CD62L<sub>high</sub> conversion was slightly faster for the Db/gp33-specific population, and 37% of this donor population had converted from CD62L<sub>low</sub> to CD62L<sub>high</sub> over 85 days compared with 27% for the Db/NP396-specific population (Fig. 5, B and C). This difference in the rate of conversion in these lineage-tracing experiments was consistent with the observations in intact unmanipulated mice in Fig. 1. For both the Db/NP396- and Db/gp33-specific CD8 T cell populations, homeostatic proliferation was enriched in the cells that had converted from CD62L<sub>low</sub> to CD62L<sub>high</sub>. Approximately 50% of the converted CD62L<sub>high</sub> population underwent homeostatic proliferation vs only ~20–25% of the cells that remained CD62L<sub>low</sub>. This observation is in good agreement with enhanced BRDU incorporation by the endogenous CD62L<sub>high</sub> Db/gp33- and Db/NP396-specific CD8 T cell populations (data not shown). These data indicate that endogenous virus-specific CD62L<sub>low</sub> TEM CD8 T cells can give rise to CD62L<sub>high</sub> TCM in the absence of Ag. In addition, the differentiation of memory CD8 T cells from CD62L<sub>low</sub> to CD62L<sub>high</sub> is accompanied by the acquisition of an enhanced ability to undergo homeostatic proliferation, a key defining property of long-lived Ag-independent memory CD8 T cells.

It has been proposed that CD62L<sub>high</sub> memory T cells are the progeny of CD62L<sub>high</sub> virus-specific T cells contained within the effector pool (27). Indeed, 2–5% of the effector CD8 T cell population in the spleen following LCMV infection (and a higher percentage in the LN) expresses CD62L<sub>high</sub> (see Figs. 1 and 2). However, the data presented above and other previous studies suggest that TEM are the product of the linear differentiation of TEM (9, 13). To examine the contribution of the small subpopulation of CD62L<sub>high</sub> day 8 LCMV-specific CD8 T cells, CD62L<sub>low</sub> LCMV-specific CD8 T cells were isolated to high purity from non-TCR transgenic C57BL/6 mice and adoptively transferred to naive mice. This experimental approach also allows one to test whether or not events occurring only during the first week of infection are sufficient to program the rate of differentiation of memory CD8 T cells.
Although 1 wk posttransfer the vast majority of the donor LCMV Db/gp33-specific CD8 T cells remained CD62Llow, by day 135 posttransfer ~65% of the Db/gp33-specific CD8 T cell population was CD62Lhigh. Moreover, when the kinetics of CD62Llow to CD62Lhigh conversion was examined the rate differentiation of this adoptively transferred purified CD62Llow donor population was nearly identical with that observed for untransferred endogenous Db/gp33-specific CD8 T cells that were CD62Lhigh at the indicated time points is also plotted and the T50% calculated. C. Homeostatic proliferation and CD62L expression were examined for the Db/gp33 donor CD8 T cells in the same mice as in B. Plots are gated on donor CD8+ Db/NP396 tetramer+ cells. The percentage of donor Db/NP396 tetramer+ CD8 T cells that were CD62Lhigh at the indicated time points is also plotted and the T50% calculated. D. Schematic of day 8 CD62Llow donor cells. CD62Llow non-TCR transgenic effector CD8 T cells were purified 8 days after LCMV Arm infection and adoptively transferred into naive recipients. The differentiation of these donor cells into the memory T cell pool was monitored. E. Purity of donor cells after magnetic bead separation was determined by comparing CD62L expression on Dbgp33- and DbNP396-specific CD8 T cells pre- and postcolumn purification. F. CD62L expression on Dbgp33-specific donor CD8 T cells at days 7 and 135 posttransfer. G. The percentage of CD62Lhigh donor Db/gp33-specific CD8 T cells is plotted at the indicated time points posttransfer. The rate of CD62L expression and T50% is shown. H. CD62L expression on Dbgp33- and DbNP396-specific CD8+ donor cells 135 days after column purification and transfer of CD62Llow day 8 donor cells.
CD8 T cells 400 days later (Fig. 6), consistent with the data presented in Fig. 5. In addition, there was a clear advantage for the CD127high fraction of the CD62Llow donor cells in forming long-lasting memory CD8 T cells (Fig. 6, B and C). Far fewer donor cells were recovered from recipients of the CD62LlowCD127low donor cells compared with recipients of the CD62LlowCD127high cells. This latter observation is consistent with previous studies demonstrating that memory CD8 T cell precursors are contained in the CD127high subset of virus-specific effector T cell populations (28). Moreover, these data make the point that whereas many TEM can convert directly to TCM, there is also a subpopulation of TEM that is not destined to enter the long-lived, self-renewing memory T cell pool. These data are consistent with phenotypic heterogeneity of the CD62Llow TEM and with a more diverse TCR repertoire in the TEM subset compared with the TCM subset (13, 14). Thus, whereas it appears that for both endogenous and TCR transgenic CD8 T cell responses the TCM CD8 T cell subset is the result of linear differentiation of TEM, at least some TEM may fail to complete this differentiation process (Fig. 6).

**Discussion**

Memory T cell subsets differ in functional properties, tissue migration, their capacity to provide protective immunity, and long-term persistence via self-renewal (9, 10, 13, 30–37). The development and lineage relationship between different memory T cell...
populations, however, remain incompletely understood. Two models have been proposed for the development of memory T cell subsets following acute infection. In the first, memory T cell subsets are unrelated lineages, arising separately early during the T cell response to acute infection or immunization (12, 15, 27, 38). According to this model, the resulting TEM and TCM subsets are regulated independently in the memory T cell pool. The second model proposes that memory T cell subsets are related lineages and that one subset converts into another over time in the absence of Ag (9, 13). In this model, TEM are a transitional population that arises between the effector phase and the development of long-lived, self-renewing TCM in the absence of Ag (9, 13). Despite intense recent interest, it has been difficult to distinguish which of these models accounts for memory CD8 T cell differentiation in vivo. In addition, regardless of which model is correct, it is important to understand and define the signals that govern the proportions of memory T cell subsets generated and the differentiation and stability of these subsets.

Using a TCR transgenic approach, we previously demonstrated that TEM were capable of converting directly into TCM in the absence of Ag in the memory pool (9, 13). Other studies have found that TEM and TCM populations can share TCR clonotypes, suggesting that these lineages have a common origin or are related (13, 14). However, recent work by Marzo et al. (15) suggests that at low precursor frequencies, such as occurs for the endogenous response to infection rather than TCR transgenic models, TEM represent a stable lineage and failed to further differentiate into TCM. In the current study, we found that the differences in memory CD8 T cell subsets and memory CD8 T cell differentiation between TCR transgenic approaches and the endogenous non-TCR transgenic response were largely kinetic. These findings are consistent with a recent report by Badovinac et al. (39). One possible caveat of some previous studies examining TEM to TCM memory T cell differentiation (15) is that these studies did not monitor differentiation over a sufficient period of time to observed TEM to TCM conversion of an endogenous CD8 T cell response. For example, if one examines TEM to TCM conversion for an endogenous CD8 T cell response to LCMV or VSV over 30 days, one would predict only 5–10% of the population would convert to CD62Lhigh based on the data in Figs. 1 and 2. This amount of conversion is quite low. In this model, TEM are a transitional population that arises between the effector phase and the development of long-lived, self-renewing TCM in the absence of Ag (9, 13). Despite intense recent interest, it has been difficult to distinguish which of these models accounts for memory CD8 T cell differentiation in vivo. In addition, regardless of which model is correct, it is important to understand and define the signals that govern the proportions of memory T cell subsets generated and the differentiation and stability of these subsets.

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Thus, our data now demonstrate that even at low precursor frequencies and low clonal competition, TEM can differentiate into TCM over time. We also define several factors that can influence the rate of memory CD8 T cell differentiation, including epitope specificity, T cell precursor frequency, clonal competition, and the duration of stimulation. In our experiments, more intense and prolonged initial T cell stimulation led to more protracted differentiation from TEM to TCM. It is important to point out that we have only examined the formation of memory CD8 T cells following primary acute infection when Ag is completely cleared. In other circumstances, such as secondary infections or when Ag persists during chronic infections, a different pattern of memory T cell differentiation may be observed (21, 40–45).

Analysis of the endogenous CD8 T cell response to three LCMV epitopes demonstrated the profound impact that early priming events can have on memory CD8 T cell differentiation over an exceptionally long period of time. For example, despite being primed in the same environment, the Db/gp33-, Db/gp276-, and Db/NP396-specific CD8 T cell populations undergo memory CD8 T cell differentiation at different rates (both CD62L up-regulation and IL-2 production), and the difference in this rate was sustained over many months. These differences were even preserved when these cells were adoptively transferred to new recipients, indicating that the rate of memory CD8 T cell differentiation is programmed by events occurring during the first week of infection. Because the NP396 epitope is most likely presented earlier and at a higher level compared with the glycoprotein epitopes (17, 18, 20), the level and/or duration of Ag stimulation may be one critical difference between the signals received by the Db/gp33-, Db/gp276-, and Db/NP396-specific T cell populations. It has been reported that T cell precursor frequency can impact T cell differentiation through competition (46–49). In this regard, a high number of TCR transgenic T cells may also influence the rate of memory T cell differentiation by limiting the magnitude or duration of Ag stimulation through T cell competition or by accelerating the clearance of infection. The end result is a faster rate of memory CD8 T cell differentiation when naive Ag-specific CD8 T cell precursor frequency is high. Similarly, shortening the duration of exposure to infection also leads to more rapid TEM to TCM differentiation. It is likely that the strength of the antigenic stimulus is an important factor governing the rate of memory CD8 T cell differentiation following acute infection. However, it is also possible that increased clonal competition and shortening the duration of exposure to infection may impact accessibility to other factors, including inflammatory cytokines, survival signals such as γ-chain cytokines, and costimulation. Given the importance of inflammatory signals in controlling more effector-like aspects of the developing CD8 T cell response (50, 51), it will be important to further distinguish the role of Ag and non-Ag signals early during priming in the differentiation of TEM to TCM.

Another interesting aspect of the current studies is that memory CD8 T cells converted from TEM to TCM in most tissues examined, including nonlymphoid tissues such as the liver. The rate of this memory CD8 T cell differentiation was different in different locations, although it is not currently known whether these differences represent biases in migration (e.g., CD62Lhigh preferentially homing to LN) or tissue-specific influences on memory T cell differentiation. Although it is clear that in some tissues, such as the brain and intestinal mucosa, memory CD8 T cells do not acquire a CD62Lhigh phenotype even after extended periods of time, it is possible that memory CD8 T cells in these locations are directly influenced by their tissue microenvironment and also may not be in dynamic equilibrium with the systemic pool of memory T cells (11, 22, 23). However, for the majority of memory CD8 T cells contained in lymphoid tissues and many nonlymphoid tissues, our observations suggest that the systemic memory CD8 T cell pool undergoes TEM to TCM differentiation.

In the early memory T cell pool, the CD62Llow population can be further subdivided based on CD127 expression. The purification and adoptive transfer of CD127high and CD127low TEM revealed that even within the TEM subset there were differences in the potential to form long-lasting memory CD8 T cells. These experiments clearly demonstrated that CD127highCD62Llow TEM could give rise to CD62Lhigh TCM in the absence of Ag. However, we also found that the CD127high TEM were more efficient at persisting and converting to TCM compared with the CD127low TEM. This observation is consistent with previous reports showing that memory T cell precursors expressed high levels of CD127 (28). However, these experiments highlight the fact that not all TEM convert to TCM, but also indicate that those TEM that do not further differentiate into TCM are not a stable population over the long-term. This result provides some explanation for observations of separate TCR clonotypes in the TEM and TCM populations (13, 14). Thus, when Ag is cleared following acute infection, it appears that the major pathway for the generation of self-renewing and stable
memory T cells ($T_{CM}$) is from the linear differentiation of $T_{EM}$. This pathway of memory T cell generation does not exclude the possibility that not all $T_{EM}$ are programmed to complete this differentiation process. As our understanding of the early events in T cell priming and differentiation improves, we may be able to more fully define the pathways regulating the generation of a population of terminal differentiated or end stage effector or $T_{EM}$ cells, and to dissect why some $T_{EM}$ progress to $T_{CM}$ and others do not. Together, our observations suggest that the effector or $T_{EM}$ phase of the T cell response is prolonged following strong stimulation. There may be an evolutionary advantage to such a scenario because a stronger stimulation will often indicate a more vigorous infection. An extended period with effector T cells or $T_{EM}$ cells may facilitate complete control of such infections or provide enhanced protection against the pathogen as it circulates in the community. However, this protracted effector-like phase comes at the enhanced protection against the pathogen as it circulates in the community. It may facilitate complete control of such infections or provide enhanced protection against the pathogen as it circulates in the community.

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Disclosures

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References


