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Increased Numbers of Preexisting Memory CD8 T Cells and Decreased T-bet Expression Can Restrain Terminal Differentiation of Secondary Effector and Memory CD8 T Cells

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Memory CD8 T cells acquire effector memory cell properties after reinfection and may reach terminally differentiated, senescent states ("Hayflick limit") after multiple infections. The signals controlling this process are not well understood, but we found that the degree of secondary effector and memory CD8 T cell differentiation was intimately linked to the amount of T-bet expressed upon reactivation and preexisting memory CD8 T cell number (i.e., primary memory CD8 T cell precursor frequency) present during secondary infection. Compared with naïve cells, memory CD8 T cells were predisposed toward terminal effector (TE) cell differentiation because they could immediately respond to IL-12 and induce T-bet, even in the absence of Ag. TE cell formation after secondary (2°) or tertiary infections was dependent on increased T-bet expression because T-bet divides cells resistant to these phenotypic changes. Larger numbers of preexisting memory CD8 T cells limited the duration of 2° infection and the amount of IL-12 produced, and consequently, this reduced T-bet expression and the proportion of 2° TE CD8 T cells that formed. Together, these data show that over repeated infections, memory CD8 T cell quality and proliferative fitness is not strictly determined by the number of serial encounters with Ag or cell divisions, but is a function of the CD8 T cell differentiation state, which is genetically controlled in a T-bet-dependent manner. This differentiation state can be modulated by preexisting memory CD8 T cell number and the intensity of inflammation during reinfection. These results have important implications for vaccinations involving priming strategies. The Journal of Immunology, 2011, 187: 000–000.

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t is widely accepted that having increased numbers of memory CD8 T cells correlates with better protection from secondary (2°) infection (1–3). However, effective vaccines and immunotherapies should not only increase memory T cell numbers but also generate the most protective and pertinent types of memory cells for a given infection. CD62Lhi CCR7hi IL-7Rhi CD28hi CD27hi central memory T cells (TCM) are commonly found in lymphoid tissues, are generally long-lived, have a high proliferative capacity, and are able to self-renew and undergo homeostatic turnover (4–9). In contrast, CD62Llo CCR7lo CD28lo CD27lo effector memory T cells (TEM) appear more differentiated because they are excluded from lymphoid tissues, vary in IL-7R expression, have a reduced ability to proliferate and produce IL-2, and are more \"effector-like\" in the resting state (4, 6, 10–14). TEM also contain less telomerase activity and have shorter telomeres than TCM (6), and many TEM express killer cell lectin-like receptor G1 (KLRG1) and CD57, two markers associated with reduced proliferative potential and senescence (15–20). As a result of their notable functional differences, the protection conferred by TCM and TEM may differ according to the type of pathogen and site of infection (4, 9, 13, 21). For example, TCM have been shown to better protect against chronic LCMV and vaccinia virus infections (13, 22). Additionally, the frequency of CD28lo TEM negatively correlates with immune responsiveness during influenza vaccination in elderly humans (23, 24). However, despite their proliferative disadvantage, several groups have reported that TEM are more protective in certain settings of infection (9, 13, 22, 25). Therefore, maintaining an optimal balance of TCM and TEM for a particular pathogen likely confers the greatest protection to the host.

Memory CD8 T cells are poised to expand rapidly and differentiate into potent 2° effector cells that swiftly control infection (7, 25, 26); however, this inherent ability to differentiate may also steer Ag-specific CD8 T cells toward terminal differentiation and senescence. For instance, studies transferring Ag-specific memory CD8 T cells into naive hosts and subsequently infecting them have shown that the resulting 2° effector and memory CD8 T cell populations consist mostly of cells with terminally differentiated TEM phenotypes (e.g., KLRG1lo IL-7Rlo CD62Llo CD27lo) that have reduced proliferative potential, although with time, less differentiated 2° memory cells can slowly reemerge (19, 25–28). Thus, it is possible that consecutive infections can drive memory CD8 T cells toward their Hayflick limit and eventually exhaust the memory pool of CD8 T cells with high proliferative fitness (15, 26).
Little is known about the signals and mechanisms that regulate the differentiation of 2’ effector and memory CD8 T cells during reinfection, but it is likely that factors that affect CD8 T cell differentiation during primary (1’) infection will be involved — such as the duration and location of infection, the frequency of precursors, and exposure to inflammatory cytokines (reviewed in Ref. 29). Certain transcription factors also regulate effector CD8 T cell differentiation, including Blimp-1, ID2, eomesodomin (eomes), and T-bet (Tbx21) for vaccine development. Generated during past and future infections impact developing vaccines against pathogens and cancer. In this study, we identify several parameters that regulate the differentiation of CD8 T cells and form CD62Lhi TCRαβ cells at a faster rate postinfection (16, 17, 30, 31). T-bet expression can be augmented by TCR, certain inflammatory cytokines (such as IL-12), and mTOR in 1’ effector CD8 T cells (17, 35–37), but the regulation of T-bet expression appears to act like a rheostat to modulate the extent to which 1’ effector CD8 T cells differentiate because increased amounts of T-bet can direct the development of terminally differentiated, senescent KLRG1hi CD8 T cells, whereas lower T-bet levels (along with eomes) are required for normal memory CD8 T cell gene expression and homeostasis (16, 17, 33, 34). Additionally, T-bet- and Blimp-1-deficient mice lack KLRG1hi IL-7Rb terminal effector (TE) CD8 T cells and form CD62Lhi TCRαβ at a faster rate postinfection (16, 17, 30, 31). T-bet expression can be increased by TCR, certain inflammatory cytokines (such as IL-12), and mTOR in 1’ effector CD8 T cells (17, 35–37), but the regulation of T-bet expression in memory CD8 T cells has not been explored.

Given the importance of memory CD8 T cells in mediating long-term protective immunity to repeated infections over the course of a lifetime, a better understanding of the signals and processes that regulate their differentiation is critical to generating more efficacious vaccines against pathogens and cancer. In this study, we identify several parameters that regulate the differentiation of memory CD8 T cells and acquisition of senescent phenotypes through successive rounds of reinfection. Our data indicate that modulating the amount of IL-12, the number of preexisting memory CD8 T cells, or the amount of T-bet they express can influence the types of 2’ or tertiary (3’) effector and memory CD8 T cells that form. This study clearly demonstrates how signals generated during past and future infections impact developing effector and memory CD8 T cells and has important implications for vaccine development.

Materials and Methods

Mice, infections, and treatments

Wild-type (Wt) and Tbx21+/−/Thy1.1+Wt Ly5.1+Wt P14 TCR transgenic mice have previously been described (8, 17). In transfer experiments, the indicated numbers of naive CD45.1+ or Thy1.1+Wt P14 CD8 T cells were transferred i.v. into naive or lymphopenic choriomeningitis virus (LCMV)-immune (30+ days postinfection) C57BL/6 mice (B6; National Cancer Institute, Fredrick, MD), Tbx21+/−×B6 F1 recipients (for experiments comparing Wt and Tbx21−/− cells), or B6.Cg129P1-P14-129P1 (B6; Jackson Laboratory, Bar Harbor, ME). In some experiments, splenic memory P14 CD8 T cells and endogenous GP33–41-specific CD8 T cells were isolated from mice 30+ days postinfection (p.i.) prior to transfer into naive recipients. In all transfer experiments, unless otherwise indicated, 0.5 × 10⁶ to 1 × 10⁷ P14 or GP33–41-specific CD8 T cells were transferred. Where indicated, CD8 T cells were labeled with CFSE (Invitrogen; Carlsbad, CA) prior to transfer. Where indicated, P14 or GP33–41-specific endogenous CD8 T cells were sorted based on surface marker expression prior to transfer or culture using a FACSAria (BD). Mice were infected i.p. with 2 × 10⁵ PFU LCMV-Armstrong or 2 × 10⁶ PFU GP33–41-expressing vaccinia virus (VV-33) or i.v. with 2 × 10⁶ CFU [unless otherwise specified] GP33–41-expressing Listeria monocytogenes (LM-33) or 2 × 10⁶ PFU LCMV-clone 13 (17). Splenic LM-33 liters were determined on day 1 p.i. as described previously (38). Ampicillin (amp) treatments 24 h p.i. were described previously (39). To deplete Thy1.1+ cells, mice received two 0.5 mg i.p. injections of Thy1.1-depleting Ab (HIS51) on alternate days. All animal experiments were performed with approved institutional animal care and use committee protocols.

Cell staining, flow cytometry, and ELISA

Lymphocyte isolations, MHC class I tetramer production, LCMV peptide stimulations, and surface/intracellular and CFSE labeling were performed as described previously (8). Anti-KLRG1 Ab was generated as described previously (17). All Abs were purchased from eBioscience (San Diego, CA) except anti-granzyme B (GzB; Invitrogen; Carlsbad, CA), anti–CXCR3 (R&D Systems; Minneapolis, MN), and Thy1.1 (BD Biosciences, San Diego, CA). T-bet staining has been described previously (17), and GzB and eomes were stained using this protocol. Flow cytometry was performed using an LSRII (BD Biosciences, San Diego, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR). Day 1 LM-33–infected mouse serum was analyzed with a mouse IL-12p70 ELISA kit (BD) according to the manufacturer’s instructions.

In vitro cultures

In vitro cultures were set up as done previously (17). Briefly, 5 × 10⁵ CD8+ P14 T cells were purified from naive P14 and LCMV-immune splenocytes by MACS using biotinylated anti-Thy1.1 (eBioscience) and streptavidin magnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions and cultured in 96-well plates with 1 × 10⁶ unlabeled or GP33–41–prelabelled B6 splenocytes with or without IL-12 (10 ng/ml; R&D Systems) for 24 h.

Telomerase assays and intracellular cytokine staining

P14 memory CD8 T cells from days 30 to 45 p.i. were sorted into the following subsets: 1) KLRG1hi IL-7Rα−CD62Llo, 2) KLRG1hi IL-7Rα−CD62Lhi, 3) KLRG1hi IL-7Rα−CD62Lhi, and 4) KLRG1−IL-7Rα−CD62Lhi. To measure the amount of telomerase, these cells were then processed according to US Biomax Quantitative Telomerase Detection Kit (US Biomax, Rockville, MD), and the amount of telomerase activity was quantitated using RT-PCR with SYBR Green. 293T cells served as a standard control, and the Ct values from the memory CD8 T cell subsets were normalized to 400 293T cell equivalents. To measure cytokine production, the sorted cells were stimulated with feeder B6 splenocytes and GP33–41 peptide for 5 h with brefeldin A. The cells were then stained for surface Thy1.1 and CD8 and intracellular IFN-γ and IL-2 as described previously (17).

Results

Increased T-bet expression is associated with terminally differentiated phenotypes in 1’ and 2’ effector and memory CD8 T cells

The heterogeneous populations of effector and memory CD8 T cells generated during LCMV infection can be dissociated into multiple subsets based on the expression of several surface receptors (such as KLRG1, IL-7R, CD27, CXCR3, and CD62L) and their associated functional attributes [such as the ability to produce IL-2, persist, and proliferate in response to 2’ infection and homeostatic cytokines (Fig. 1A, Supplemental Figs. 1–3, and Refs. 7, 15, 17–19, 27, 40, 41)]. When ordered according to the amount of T-bet expressed by each subset, a spectrum of differentiation states emerged in 1’ effector and memory populations (Fig. 1A). For the most part, expression of T-bet and eomes was inversely correlated, and cells expressing higher amounts of T-bet expressed lower amounts of eomes (Fig. 1A [note the T-bet/eomes ratio]; Supplemental Fig. 1). T-bet expression was highest in effector CD8 T cells 8 d p.i. but progressively declined as memory CD8 T cells formed (Fig. 1A, 1C). Conversely, eomes expression increased during the effector → memory cell transition. Notably, a functional hierarchy was observed that correlated with the CD8 T cell differentiation state and the T-bet/eomes expression ratio when we compared the four most represented memory subsets (analyzed 30–60 d p.i.): No. 1, KLRG1hi IL-7Rα−CXCR3hi CD62Llo; No. 2, KLRG1hi IL-7Rα−CXCR3hi CD62Lhi; No. 3, KLRG1−IL-7Rα−CXCR3hi CD62Llo; No. 4, KLRG1−IL-7Rα− CXCR3−CD62Lhi (note that CD27 and CXCR3 displayed nearly overlapping expression patterns). Examination of cytokine production revealed that the subsets produced similar amounts of IFN-γ, however, the ability to produce IL-2 was greatest in subset No. 1 followed by subsets No. 2, No. 3, and No. 4 (Supplemental Fig. 3). Likewise, a similar hierarchy of proliferative responses to
2˚ infection was observed (No. 1 = No. 2 = No. 3 = No. 4) (Supplemental Fig. 3). Lastly, telomerase activity was higher in KLRG1lo IL-7Rhi CXCR3hi CD62Lhi (TCM) cells compared with that in KLRG1lo IL-7Rhi CXCR3lo CD62Llo or KLRG1hi IL-7Rhi CXCR3lo CD62Llo (TEM) cells (14, 42, 43) but surprisingly the levels of telomerase activity in the KLRG1hi IL-7Rlo CXCR3lo CD62Llo TEM memory cell subset was marginally higher than the TCM (Supplemental Fig. 3).

As seen prior, KLRG1hi IL-7Rlo CXCR3lo CD62Llo cells, which represented the largest effector cell subset, had reduced longevity and gradually declined over time in the spleen (Fig. 1B, Supplemental Fig. 2) (8, 17, 18, 44). In contrast, the KLRG1hi IL-7Rhi CXCR3hi CD62Lhi (TCM) cells accumulated in the memory CD8 T cell population due to enhanced survival and homeostatic proliferation. Thus, the KLRG1lo IL-7Rlo CXCR3hi CD62Llo TCM contain the most polyfunctional cells with the greatest longevity and proliferative capacity to 2˚ infection and homeostatic cytokines; these data are consistent with previous reports (7, 8, 10, 11, 13, 17, 18, 41, 45, 46). Because of these functional differences, we consider cells bearing a KLRG1hi IL-7Rlo CXCR3lo CD62Llo TEM phenotype to be the most senescent and terminally differentiated relative to the other subsets.

Next, we examined the formation of these phenotypically and functionally distinct effector and memory CD8 T cell subsets during the course of a 2˚ infection. Consistent with previous reports, after adoptive transfer and reinfection with recombinant *Listeria* expressing GP33–41 (LM-33), the 2˚ effector and memory CD8 T cell populations in the spleen were composed of cells expressing a more differentiated phenotype with increased expression of KLRG1 and GzB and lower expression of IL-7R, CD27, CXCR3, CD62L, and IL-2 (Fig. 1B, data not shown) (25–27). Correspondingly, the 2˚ effector and memory CD8
T cells expressed higher amounts of T-bet and lower amounts of eomes than 1˚ effector or memory CD8 T cells after infection (Fig. 1A, 1C), but over several months, cells of a lesser differentiated phenotype dominated the 2˚ memory CD8 T cell population in the spleen (Fig. 1B, Supplemental Fig. 2). However, 2˚ memory cells retained some characteristics of their more differentiated state including reduced IL-2 production and increased GzB (Fig. 1D). Thus, compared with 1˚ infection, 2˚ effector and memory CD8 T cells more efficiently adopt and retain terminally differentiated TEM phenotypes.

Reducing T-bet impairs terminal differentiation of 2˚ and 3˚ effector CD8 T cells

Possibly, memory CD8 T cells are prone to acquiring terminally differentiated states upon 2˚ infection because they upregulate T-bet more rapidly and to a higher degree than naive CD8 T cells. On the one hand, this might enhance protection by promoting rapid development of effector cells upon reinfection, but on the other hand, this may come with an inherent cost by limiting their proliferative capacity (19, 22). Therefore, we tested whether genetically reducing T-bet expression by one-half affected the differentiation of 2˚ and 3˚ effector and memory CD8 T cells and their ability to respond to serial infections (see experimental outline in Fig. 2A). First, we transferred small numbers of naive T-bet Wt (Tbx21+/+) or heterozygous (Tbx21+/−) P14 CD8 T cells into B6 mice and infected them with LCMV. After more than 50 d, the Wt and Tbx21+/− 1˚ memory populations were mostly made up of KLRG1hi IL-7Rlo CD27lo CXCR3hi CD8 T cells, and many cells expressed CD62L and produced IL-2, albeit these types of memory CD8 T cells were more prevalent in Tbx21+/− memory CD8 T cells (Fig. 2A, Supplemental Fig. 4A, 4E). Wt and Tbx21+/− memory CD8 T cells also had similar rates of homeostatic turnover (data not shown).

Next, we compared the ability of Wt and Tbx21+/− memory P14 CD8 T cells to differentiate into 2˚ effector and memory CD8 T cells by transferring equal numbers of the two populations into naive recipients and infecting them with LM-33. Interestingly, the Tbx21+/− 2˚ effector CD8 T cell population continued to express one-half the amount of T-bet as Wt cells during the 2˚ infection (Fig. 2B). Nearly all the Wt 2˚ effector and memory CD8 T cells acquired a KLRG1hi IL-7Rlo CD27lo CXCR3hi phenotype, whereas in contrast, a substantial number of Tbx21+/− 2˚ effector and memory CD8 T cells retained a KLRG1lo IL-7Rhi CD27hi CXCR3hi phenotype (Fig. 2A, Supplemental Fig. 4B). The Wt 2˚ memory CD8 T cells also expressed more GzB and produced less IL-2 than the Tbx21+/− 2˚ memory CD8 T cell population (Supplemental Fig. 4E). Given the diminished ability of Tbx21+/− CD8 T cells to acquire terminally differentiated

FIGURE 2. Limiting T-bet expression can reduce terminal differentiation and sustain proliferative capacity of memory CD8 T cells after successive infections. A–C, 1 × 10^4 Wt or Tbx21+/− naive, 1˚ or 2˚ memory P14 CD8 T cells were transferred into naive mice that were subsequently infected with LCMV (1˚ infection), LM-33 (2˚ infection), or VV-33 (3˚ infection), respectively. At the indicated time points after 1˚, 2˚, or 3˚ infection, the donor Thy1.1+ P14 CD8 T cells were enumerated, and KLRG1, IL-7R, CXCR3, CD62L, T-bet, and eomes expression was analyzed. A, Pie charts show the frequency of 1˚ memory, 2˚ effector, 2˚ memory, and 3˚ effector CD8 T cells in the subpopulations determined based on KLRG1, IL-7R, CXCR3, and CD62L expression (see Fig. 1A). For more phenotypic data, see Supplemental Fig. 4. B, Histograms show T-bet and eomes expression in Wt (line) and Tbx21+/− (shaded) 1˚, 2˚, and 3˚ effector CD8 T cells. Note, at all time points, expression of T-bet was ~2- to 3-fold lower and eomes was ~2-fold higher in Tbx21+/− CD8 T cells compared with those in Wt cells. C, Bar graphs show the number (×10^5) of P14 1˚, 2˚, and 3˚ effector and memory CD8+ T cells at 7 and 50+ d, respectively, p.i. Data are representative of five to seven experiments.
CD8 T cells were transferred into naive recipients that were subsequently infected with LM-33. Seven days later, donor splenocytes were analyzed. In contrast to Wt mice, substantially fewer 3˚ effector CD8 T cells continued to express more eomes and less T-bet than the Wt cells (Fig. 2A). The clonal burst of the Tbx21+/− cells during the first or second infection was equal to or reduced compared with the Wt cells, but during the third infection the number of 3˚ Tbx21+/− effector CD8 T cells outnumbered the Wt cells by ~2.5-fold (Fig. 2C). Together, these data show that the differentiation states of memory CD8 T cells are not strictly controlled by the number of cell divisions or infections a cell encounters, but depend on increased T-bet expression. Thus, limiting T-bet expression may maintain memory CD8 T cells in less differentiated states and preserve their proliferative capacity across successive waves of infection.

Memory CD8 T cells are more receptive to inflammatory signals compared with naive T cells

Compared with naive CD8 T cells, memory CD8 T cells may be more prone to acquiring TE and TEM states after infection because they express higher amounts of certain inflammatory cytokine receptors, such as IL-12Rβ2. IL-12 is particularly important for T-bet expression and 1˚ TE and TEM CD8 T cell formation during Listeria infection (17, 22, 47–50), so we next tested whether IL-12 deficiency affected the types of 2˚ effector CD8 T cells that formed during 2˚ Listeria infection. Memory CD8 T cells were transferred into Wt and IL-12–deficient (Il12p40−/−) recipients, which were subsequently challenged with LM-33. Seven days later, donor splenocytes were analyzed. In contrast to Wt mice, substantially fewer 2˚ effector CD8 T cells acquired KLRG1hi IL-7Rlo CD27lo CXCR3lo phenotype in the IL-12–deficient mice, and they expressed less T-bet (Supplemental Fig. 5).

Next, we directly compared the IL-12 responsiveness between naive and memory CD8 T cells in vitro. As expected from previous work, T-bet was not induced within 24 h in naive CD8 T cells, under any condition, but was upregulated after 48 h with peptide or peptide plus IL-12 (Fig. 3A, data not shown, and Ref. 17). Conversely, resting memory CD8 T cells expressed T-bet, and within 24 h, T-bet expression was augmented considerably in memory CD8 T cells stimulated with either IL-12 plus peptide or IL-12 alone (Fig. 3A).

Lastly, we determined whether the memory CD8 T cell hypersensitivity to IL-12 affected 2˚ effector CD8 T cell differentiation in vivo. To address this question, we truncated Listeria infection with early antibiotic treatment (which blunts the amount of IL-12 and IFN-γ produced during the first 24 h) in two groups of mice containing equal numbers (5 × 10^5) of Thy1.1° naive or memory P14 CD8 T cells (49, 51). As shown previously, amp treatment during priming of naive CD8 T cells profoundly reduced the amount of T-bet expressed and resulted in substantially fewer KLRG1hi IL-7Rlo CD27lo CXCR3lo 1˚ effector CD8 T cells (Fig. 3B and Refs. 17, 39, 51, 52). In contrast, amp treatment was less effective at perturbing the priming of memory CD8 T cells and differentiation of 2˚ effector CD8 T cells. These results are consistent with the idea that immediately upon infection, even in the absence of Ag, memory CD8 T cells are more poised than naive cells to respond to inflammatory signals and further differentiate.

**Increased precursor frequency of memory CD8 T cells prevents further differentiation of 2˚ effector CD8 T cells**

We reasoned that another important parameter that could affect 2˚ effector and memory CD8 T cell differentiation was the number of preexisting memory CD8 T cells in immune animals (i.e., memory CD8 T cell precursor frequency) because the precursor frequency of Ag-specific memory CD8 T cells is naturally high in immune animals. To examine this point, we devised three different experimental systems wherein the number and precursor frequency of GP33–41-specific memory CD8 T cells was varied across a dynamic range to assess how 2˚ effector and memory CD8 T cell differentiation during 2˚ LM-33 infection would be affected (Fig. 4).

In the first system, we transferred 5 × 10^3 GP33–41-specific (non-Tg) memory CD8 T cells from Thy1.1° LCMV-immune mice into Thy1.2° naive B6 or LCMV-immune mice, which naturally contain ~1.5 × 10^5 to 5 × 10^5 GP33–41-specific CD8 T cells. This created two groups of mice in which the numbers of GP33–41-specific CD8 T cell memory cell precursors differed more than

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**FIGURE 3.** Memory CD8 T cells are poised to respond to IL-12 and upregulate T-bet. A, MACS-purified naive (shaded) or 1˚ memory (line) P14 CD8 T cells were incubated with either peptide-pulsed or unpulsed splenocytes with or without IL-12 in vitro and analyzed for T-bet expression 24 h later. B, 1 × 10^6 naive or memory Thy1.1° CD8° P14 CD8 T cells were transferred into naive recipients that were subsequently infected with LM-33. After 24 h, mice were either treated with amp (+ Amp) or left untreated (No Amp). FACS plots show the expression of KLRG1 and IL-7R (middle panels) or T-bet (right panels) on transferred Thy1.1° CD8° splenocytes. Pie charts show the frequency of 1˚ effector or 2˚ effector CD8 T cells in the subpopulations determined based on KLRG1, IL-7R, CXCR3, and CD62L expression (see Fig. 1A). Data are representative of two to three experiments.
and similar expression of T-bet (for details on methodology, see Supplemental Fig. 7). Seven days after 1° LCMV infection and, consequently, similar expression of T-bet (combination of red and orange groups) was representative of three to five experiments. *p < 0.05.

Increased numbers of preexisting memory CD8 T cells curtail production of IL-12 and 2° effector CD8 T cell differentiation during Listeria infection

It is most certain that the above effects on 2° effector CD8 T cell differentiation were observed in part because LCM3-33 was more rapidly controlled by increased numbers of memory CD8 T cells (10, 13, 17, 39, 47, 51–62). Indeed, at day 1 p.i., the LM-33–infected LCMV-immune recipients had >100-fold lower bacterial titers and sharply reduced levels of IL-12p70 compared with those of LM-33–infected naive recipients (Fig. 5A, 5B). However, these differences were somewhat normalized when LCMV-immune recipients were infected with 100-fold more LM-33 (2 × 10⁶ CFU; “LM-33-High”; Fig. 5A, 5B), which also led to an increased percentage of KLRG1hi IL-7Rlo 2° effector CD8 T cells in the LCMV-immune mice (Fig. 5C). Similarly, infection of naive recipients with 100-fold less LM-33 (2 × 10⁴ CFU; “LM-33-Low”) resulted in a decreased percentage of KLRG1hi IL-7Rlo 2° effector CD8 T cells, which was rescued by treatment with CpG-B (a potent IL-12 inducer) (Fig. 5D). Collectively, these data strongly support the model that T-bet expression and terminal differentiation of 2° effector CD8 T cells is sensitive to the amount of inflammation and IL-12 present during 2° infection, and that the naturally high number of memory CD8 T cells present in immune animals “protects” the cells from acquiring terminally

FIGURE 4. The number of preexisting memory CD8 T cells influences the differentiation state of 2° effector CD8 T cells. A–D, 5 × 10⁴ unlabeled (A, B) or 10³, 10⁴, and 10⁵ CFSE-labeled (C, D) Thy1.1⁺ P14 memory CD8 T cells from day 50+ p.i. were transferred into naive or LCMV-immune recipients (Thy1.2⁺) that were subsequently infected with LM-33. E and F, LCMV-immune mice containing a range of P14 memory CD8 T cells (between ~2 × 10⁴ to 5 × 10⁵ and ~1.5 × 10⁶ cells and comprising between 0.2 and 10% of the total CD8 T cell population) were generated as described in Supplemental Fig. 7 and rechallenged with LM-33. A, C, and E, Pie charts show the frequency of different 2° effector CD8 T cell subsets based on KLRG1, IL-7R, CXCR3, and CD62L expression 7 d p.i. Note that cell numbers and phenotypic data are shown in Supplemental Fig. 6. B, D, and F, Bar graphs show T-bet expression (based on MFI) in the 2° effector CD8 T cells of the indicated recipients. Note that in C and D, only recruited (CFSEneg) cells were analyzed. Data are representative of three to five experiments. *p < 0.05.
differentiated, senescent states by severely limiting both the duration of infection and the production of inflammatory cytokines.

Discussion
Memory CD8 T cells remain poised for reactivation and rapid differentiation into 2nd effector CD8 T cells, which enables a swift and protective immune response upon pathogen reinvasion. However, memory T-cell differentiation does not stop after the first encounter with Ag. During subsequent encounters with Ag, memory CD8 T cells can further differentiate into cells that more stably persist in TEM states and have a reduced ability to proliferate, self-renew, and, depending on the nature of the pathogen, protect against future infection (63, 64). In this study, we aimed to identify parameters that affect the differentiation of 2nd effector and memory CD8 T cells upon reinfection because little is known about this process.

During the effector→memory CD8 T-cell transition, T-bet expression declines, but this low-level expression sustains the expression of the IL-2/15R β-chain (CD122), which supports memory CD8 T-cell longevity and self-renewal (17, 65). As shown in this study, memory CD8 T cells more rapidly upregulate T-bet, relative to naive T cells, and this is needed for memory CD8 T cells to differentiate further and acquire terminally differentiated features during 2nd, or even 3rd, infection. It is likely that epigenetic changes in the transcriptional regulation of T-bet contribute to its rapid re-expression in memory CD8 T cells, but we have shown that an additional cause stems from greater IL-12 responsiveness due to sustained expression of IL-12Rβ2, and this leads to more rapid induction of T-bet expression, even in the absence of antigenic stimulation. Additionally, memory CD8 T cells also express other inflammatory cytokine receptors that could induce T-bet at steady state, including IFN-αR, IFN-γR, IL-18R, and IL-27R (49, 66–69). These results suggest that early exposure of memory CD8 T cells to inflammatory cytokines upon infection can increase T-bet expression, even prior to TCR stimulation, to augment the effector functions and burst of 2nd effector CD8 T cells. This aspect also raises the possibility that bystander inflammation could influence the differentiation state and function of “resting” memory CD8 T cells.

Most studies examining the phenotypes of 2nd effector CD8 T cells have been performed by adoptively transferring small numbers of memory CD8 T cells into naive recipients and rechallenging them with 2nd infections (10, 12, 13, 20, 26). These adoptive transfer experiments demonstrated the tendency of memory CD8 T cells to acquire TEM phenotypes upon reinfection, and in some cases these 2nd TEM provide greater immunity to certain infections (15, 25, 26, 70). These results may represent what occurs in hosts where relatively low numbers of memory CD8 T cells exist; however, in immune animals the precursor frequency of Ag-specific memory CD8 T cells is naturally high, and therefore these transfer experiments likely overestimate the degree to which 2nd memory CD8 T cells differentiate during a natural 2nd infection (26). In this study, we show that the physiologic increase in memory CD8 T-cell numbers formed by prior infection benefit the host directly by causing swift removal of an invading pathogen, but also indirectly by blunting exposure of memory CD8 T cells to antigenic and inflammatory signals that augment T-bet expression and terminal differentiation. We propose that this acts, in essence, as a cellular form of “herd immunity” to preserve a proportion of memory T cells in lesser differentiated states with greater proliferative fitness. Conceptually, and likely mechanistically, this is similar to how superphysiologic numbers of Ag-specific naive CD8 T cells can reduce the percentage of TE cells and TEM that form during 1st infections (10, 17, 53). Thus, for pathogens that are serially encountered throughout life, the function, fate, and longevity of CD8 T-cell immunity may be most affected by the starting precursor frequency of the memory CD8 T-cell pool and the rate at which 2ndfections are curtailed.

Previous work has demonstrated that CD8 T cells pushed through multiple rounds of antigenic stimulation upregulate...
KLRG1 expression and lose proliferative capacity (15). This has led to the notion that excessive cell division promotes terminal differentiation and senescence, but our work in this study reveals that the acquisition of terminally differentiated and senescent states in Ag-specific CD8 T cells does not operate by a simple “clockwork mechanism” based on the number of cell divisions or encounters with Ag over time. Rather, this process appears to depend heavily on the amount of T-bet (and likely other transcriptional regulators) expressed by reactivated memory CD8 T cells that directly control differentiation of effector and memory CD8 T cells after reinfecction. Notably, T-bet concentrations did not accumulate to Wt levels in the Tbx2a+/− CD8 T cells despite three waves of infection and clonal expansion, and this permitted the CD8 T cells to retain “less differentiated” phenotypes and a greater proliferative potential. CD8 T cells with limited T-bet expression may prevent memory CD8 T cells from becoming senescent and could be useful in generating more durable memory CD8 T cell populations after some vaccinations.

Additionally, KLRG1+ Tem are associated with reduced telomerase activity and shortened telomeres, implying an approach of the Hayflick limit; however, KLRG1 does not appear to inhibit telomerase activity directly (42). Our data demonstrate that Tcm also have greater telomerase activity relative to other Tem subsets (KLRG1lo IL-7Rhi CXCR3lo CD62Llo and KLRG1hi IL-7Rlo CXCR3lo CD62Lhi), but surprisingly, we found the KLRG1hi IL-7Rlo CXCR3lo CD62Lhi memory CD8 T cells contained telomerase activity. Given that telomerase activity is quite high in recently activated effector CD8(+) T cells (72, 73), it is possible that these KLRG1hi IL-7Rlo CXCR3lo CD62Lhi cells contain telomerase activity because they actually are recently activated effector cells from contact with inflammatory cytokines that promote effector cell differentiation or Ag depots, despite clearance of replicating viruses.

Most studies of memory CD8 T cells have focused on the protective advantage afforded by increased memory cell numbers. We have shown in this study that increasing memory CD8 T cell numbers affects not only the rate of pathogen clearance but also the extent to which 2nd effector CD8 T cells differentiate during rechallenge. This point may be most relevant in the setting of infection. If a vaccine generates fairly small numbers of memory CD8 T cells, then these cells could be particularly prone to terminal differentiation upon 2nd boosters. Coupled with the fact that terminally differentiated CD8 T cells are typically shorter lived but can provide some protection from reinfection, this may provide an alternative explanation for why certain prime-boost vaccination strategies wane with time. Therefore, generating numbers affects not only the rate of pathogen clearance but also the extent to which 2nd effector CD8 T cells differentiate during rechallenge.

References


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Supplementary Figure Legends

Figure S1. Diversity amongst effector and memory CD8 T cells. FACS plots are gated on effector (day 8; left) and memory (day 120+; right) Thy1.1⁺ CD8⁺ T cells from LCMV-infected P14 chimeric mice. First the cells were gated according to KLRG1 and IL-7R expression (I-IV) and then CXCR3 and CD62L expression. For each detectable subset (>1% of total), the amount of T-bet and eomes in the gated cells are shown. BD= Below detection for populations <1% of the total Thy1.1⁺ CD8⁺ population.

Figure S2. Kinetics of the various CD8 T cell subpopulations during primary and secondary infection. Line graphs show the number of Thy1.1⁺ CD8⁺ P14 T cells during 1° LCMV or 2° LM-33 infection from the indicated subpopulation (colored lines) or the total (dashed lines). For each plot, the fold change from day 8 (1°) or day 7 (2°) is indicated. Note, the total fold-decrease for the 1° and 2° CD8 T cell response was 7x and 6x respectively.

Figure S3. Correlations between memory CD8⁺ T cell phenotype and functionality. A and B, P14 chimeric mice were infected with LCMV and 50+ days later, Thy1.1⁺ CD8⁺ P14 T cells were FACS sorted as indicated. A, Sorted cells were incubated with Thy1.2⁺ with and without GP33-41 peptide. FACS plots show IL-2 and IFNγ expression by Thy1.1⁺ CD8⁺ cells based on intracellular cytokine staining. B, Sorted cells were transferred to naïve Thy1.2⁺ B6 recipients that were subsequently infected with 2x10⁶ PFU of the clone13 strain of LCMV i.v. Bar graph shows the number of donor Thy1.1⁺ splenocytes recovered 7 days p.i. C, Telomerase activity was measured in naïve CD8⁺ T cells or the indicated memory CD8⁺ T cell subsets using an RT-PCR based method and the amount of telomerase activity in CD8⁺ T cells (per 400 cell equivalents) was normalized to the standard (293T cells). Bar graph shows the fold difference of telomerase activity relative to the standard (x10³) from 2 independent sets of sorted samples tested in duplicate.

Figure S4. Lowering T-bet expression in 2° and 3° effector and memory cells limits TE differentiation and proliferative senescence. Cells were transferred and mice were infected and analyzed as described in Fig. 2A. A-D, FACS plots show expression of KLRG1 and IL-7R (left panels), CXCR3 and CD62L (middle panels) or CD27 and IL-7R (right panels) on the transferred Wt (top rows) and Tbx21⁺⁻ (bottom rows) 1° memory
(A), 2° effector (B), 2° memory (C) and 3° effector (D) Thy1.1+ CD8+ T cells. Plots correspond to pie charts shown in Fig. 2A. E, Histograms show Granzyme B (GzB) and IL-2 production by 1° and 2° Wt (line) or T-bet+/- (shaded) Thy1.1+ memory CD8+ T cells.

Figure S5. IL-12 enhances 2° effector differentiation during 2° LM infection. 5x10^3 Thy1.1+ CD8+ 1° memory P14 T cells were transferred into Wt (line) or Il12p40-/-(shaded) recipients that were subsequently infected with LM-33. Seven days later the donor Thy1.1+ CD8+ T cells were analyzed for KLRG1 and IL-7R or T-bet expression by flow cytometry. Pie charts show the frequency of cells in the subpopulations based on KLRG1, IL-7R, CXCR3, and CD62L expression.

Figure S6. Increasing memory cell numbers protects 2° effector CD8 T cells from further differentiation. FACS plots (A, B and D) show KLRG1 and IL-7R expression on 2° effector CD8 T cells and bar graphs (C and E) show number of 2° effector CD8+ T cells from Fig. 4A,B (A), Fig. 4C,D (B and C) and Fig. 4E,F (D and E). B, “Uninfected” mice received 1x10^5 CFSE+ CD8+ memory T cells, but were not infected with LM-33. Based on the number of remaining CFSE+ memory cells between LM-33 infected and uninfected controls, 67-75% of the 10^5 transferred memory cells were recruited into the response to LM-33 (CFSE-), and >99% of all the Thy1.1+ 2° effector CD8 T cells in LM-33 infected mice were CFSE-.

Figure S7. Generating mice with increasing numbers of memory CD8+ T cells. A, Schematic outline for the generation of mice with varying numbers of Ly5.1+ CD8+ P14 memory T cells ranging from 100% to 50% to 10% to 1% of a typical P14 memory-chimeric mouse. A total of 2.5x10^3 CD8+ P14 T cells were transferred in various ratios of Thy1.1+: Ly5.1+ P14 CD8 T cells (0:100, 50:50, 90:10, 99:1) into different groups of B6 that were subsequently infected with LCMV. After 90 days, mice were treated twice i.p. with 0.5mg anti-Thy1.1 depleting antibody (HIS51) to deplete Thy1.1+ CD8+ T cells. Two weeks later (day 104 pi), mice were infected with LM-33, and analyzed 7 days later. B, FACS plots show the frequency of Ly5.1+ and Thy1.1+ P14 CD8+ T cells in prior to injection, ~90 days pi (peripheral blood, PBL) and 2 weeks post depletion (day ~100; PBL). C, KLRG1 and IL-7R expression on Ly5.1+ CD8+ T cells at day 90 pi. D, FACS plots show the frequency of Ly5.1+ CD8+ T cells present in the peripheral blood of mice after Thy1.1 depletion and prior to LM-33 infection. The percentage of the total CD8+ T
cell population that is comprised by the Ly5.1⁺ CD8⁺ T cells is indicated. Note, after infection, all mice had similar numbers of total Ly5.1⁺ 2° effector CD8 T cells except the mice that began with Ly5.1⁺ P14 cells as 0.2% of total CD8 T cells. In these mice, the endogenous memory cells made up 50% of the total 2° effector CD8 T cell population.
Figure S1

Effector CD8 T cells

Memory CD8 T cells

I

II

III

IV

KLRG1

IL-7R

T-bet

Eomes

KLRG1lo IL-7Rhi CXCR3hi CD62Lhi

KLRG1lo IL-7Rhi CXCR3hi CD62Llo

KLRG1lo IL-7Rlo CXCR3hi CD62Llo

KLRG1lo IL-7Rlo CXCR3lo CD62Llo

KLRG1hi IL-7Rhi CXCR3lo CD62Llo

KLRG1hi IL-7Rhi CXCR3hi CD62Lhi

KLRG1hi IL-7Rlo CXCR3hi CD62Llo

KLRG1hi IL-7Rlo CXCR3lo CD62Llo

KLRG1lo IL-7Rlo CXCR3lo CD62Llo

KLRG1lo IL-7Rhi CXCR3hi CD62Llo

KLRG1hi IL-7Rlo CXCR3hi CD62Llo

KLRG1hi IL-7Rlo CXCR3lo CD62Llo

KLRG1lo IL-7Rlo CXCR3lo CD62Llo

KLRG1lo IL-7Rhi CXCR3hi CD62Llo

KLRG1hi IL-7Rlo CXCR3hi CD62Llo

KLRG1hi IL-7Rlo CXCR3lo CD62Llo

KLRG1lo IL-7Rlo CXCR3lo CD62Llo

CD62L

CXCR3

Figure S1
**Figure S2**

Diagram showing changes in cell number over time post-infection with LCMV. Graphs illustrate different cell populations (KLRG1^lo^ IL-7R^hi^ CXCR3^hi^ CD62L^hi^, KLRG1^lo^ IL-7R^hi^ CXCR3^hi^ CD62L^lo^, KLRG1^hi^ IL-7R^hi^ CXCR3^hi^ CD62L^lo^, KLRG1^hi^ IL-7R^lo^ CXCR3^hi^ CD62L^lo^, KLRG1^lo^ IL-7R^lo^ CXCR3^lo^ CD62L^lo^, KLRG1^hi^ IL-7R^lo^ CXCR3^lo^ CD62L^lo^, KLRG1^hi^ IL-7R^hi^ CXCR3^lo^ CD62L^lo^, KLRG1^hi^ IL-7R^hi^ CXCR3^lo^ CD62L^lo^) and the fold change in cell number compared to baseline.

**1st LCMV infection**

- KLRG1^lo^ IL-7R^hi^ CXCR3^hi^ CD62L^hi^: \( \uparrow 16x \)
- KLRG1^lo^ IL-7R^hi^ CXCR3^hi^ CD62L^lo^: \( \downarrow 9x \)
- KLRG1^hi^ IL-7R^hi^ CXCR3^hi^ CD62L^lo^: \( \downarrow 32x \)
- KLRG1^lo^ IL-7R^lo^ CXCR3^hi^ CD62L^lo^: \( \downarrow 60x \)
- KLRG1^lo^ IL-7R^lo^ CXCR3^lo^ CD62L^lo^: \( \uparrow 5x \)
- KLRG1^lo^ IL-7R^lo^ CXCR3^lo^ CD62L^lo^: \( \uparrow 15x \)
- KLRG1^hi^ IL-7R^hi^ CXCR3^lo^ CD62L^lo^: \( \downarrow 226x \)
- KLRG1^hi^ IL-7R^hi^ CXCR3^lo^ CD62L^lo^: \( \uparrow 7x \)

**2nd LM-33 infection**

- KLRG1^lo^ IL-7R^hi^ CXCR3^hi^ CD62L^hi^: \( \uparrow 16x \)
- KLRG1^lo^ IL-7R^hi^ CXCR3^hi^ CD62L^lo^: \( \downarrow 2x \)
- KLRG1^hi^ IL-7R^hi^ CXCR3^hi^ CD62L^lo^: \( \downarrow 3x \)
- KLRG1^lo^ IL-7R^lo^ CXCR3^hi^ CD62L^lo^: \( \downarrow 162x \)
- KLRG1^lo^ IL-7R^lo^ CXCR3^hi^ CD62L^lo^: \( \uparrow 7x \)
- KLRG1^lo^ IL-7R^lo^ CXCR3^lo^ CD62L^lo^: \( \downarrow 571x \)
- KLRG1^lo^ IL-7R^lo^ CXCR3^lo^ CD62L^lo^: \( \downarrow 745x \)
- KLRG1^hi^ IL-7R^hi^ CXCR3^lo^ CD62L^lo^: \( \downarrow 250x \)
Figure S3

A

+ GP33-41

No Stim

KLRG1lo
IL-7Rhi
CXCR3hi
CD62Lhi

KLRG1lo
IL-7Rhi
CXCR3hi
CD62Llo

KLRG1hi
IL-7Rhi
CXCR3lo
CD62Llo

KLRG1hi
IL-7Rlo
CXCR3lo
CD62Llo

#1

#2

#3

#4

B

2° recall response

Unsorted (total) P14 memory CD8 T cells

KLRG1lo
IL-7Rhi
CXCR3hi
CD62Lhi

KLRG1lo
IL-7Rhi
CXCR3hi
CD62Llo

KLRG1hi
IL-7Rhi
CXCR3lo
CD62Llo

KLRG1hi
IL-7Rlo
CXCR3lo
CD62Llo

C

Telomerase activity

Fold difference relative to standard (x 10^3)

naive P14 CD8 T cells

KLRG1lo
IL-7Rhi
CXCR3hi
CD62Lhi

KLRG1lo
IL-7Rhi
CXCR3hi
CD62Llo

KLRG1hi
IL-7Rhi
CXCR3lo
CD62Llo

KLRG1hi
IL-7Rlo
CXCR3lo
CD62Llo

Figure S3
Figure S5
Figure S6

A

Naive Recipient | Immune Recipient

Day 7 pi

KLRG1 | IL-7R | KLRG1 | IL-7R

Day 7 pi

KLRG1 | IL-7R | KLRG1 | IL-7R

B

1x10^3 Cells | 1x10^4 Cells | 1x10^5 Cells | 1x10^6 Cells | Uninfected

Day 7 pi

KLRG1 | IL-7R | KLRG1 | IL-7R

Day 7 pi

KLRG1 | IL-7R | KLRG1 | IL-7R

C

Cells transferred

10^3 | 10^4 | 10^5

Cell Number

10^4 | 10^5 | 10^6 | 10^7 | 10^8

Total | KLRG1hi | IL-7Rlo | KLRG1lo | IL-7Rhi

D

Precursor frequency of memory CD8 T cells:

Day 7 pi

KLRG1hi | IL-7Rlo | KLRG1lo | IL-7Rhi

Day 7 pi

KLRG1hi | IL-7Rlo | KLRG1lo | IL-7Rhi

E

Frequency of CD8 T cells

0.2% | 2% | 6% | 10%

Cell Number

10^5 | 10^6 | 10^7

Total | KLRG1hi | IL-7Rlo | KLRG1lo | IL-7Rhi

Figure S6
(1) Mix Thy1.1+ and Ly5.1+ P14 naïve CD8 T cells at different ratios

Transfer 2.5x10^3 total P14 cells

Day: 0 90+2 104 104 +7

Day ~90 (Pre-Depletion)

Day ~100 (Post-Depletion)

Ly5.1

CD8

Thy1.1

100% 99% 100% 100% 100% 0%

50% 41% 55% 0% 4% 32%

10% 58% 45% 2% 21% 27%

1% 12% 6% 3% 19% 25%

0% 0% 0% 0%

25% 40% 4% 32% 6% 4%

Figure S7