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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+ cells were 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 Ag-specific memory CD8 T cells for a given infection. CD62Lhi CCR7hi IL-7Rhi TEMbers but also generate the most protective and pertinent types of memory CD8 T cells. These data also indicate that the balance of 2°/3° memory cells is not solely determined by the magnitude of Ag-specific memory CD8 T cells but also contains 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 naïve 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., KLRG1hi 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, eomesoderm (eomes), and T-bet (Tbx21) (16, 17, 30–33). In particular, 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 TCM 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 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 states 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+ and Wt Ly5.1+ P14 TCR transgenic mice have previously been described (8, 17). In transfer experiments, the indicated numbers of naive CD45.1+ or Thy1.1+ P14 CD8 T cells were transferred i.v. into naive or lymphocytic 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 B120p40+ mice (The 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 × 10⁷ to 1 × 10⁸ 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 × 10⁶ 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 titers 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 LCMV–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 × 10⁶ 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 incubated in 96-well plates with 1 × 10⁶ unlabeled or GP33–41-prefield 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-7Rhi CD62Llo, 2) KLRG1lo IL-7Rhi CD62Llo, 3) KLRG1lo IL-7Rlo CD62Lhi, and 4) KLRG1lo IL-7Rlo 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, KLRG1lo IL-7Rhi CXCR3lo CD62Lhi; No. 2, KLRG1lo IL-7Rlo CD62Lhi; No. 3, KLRG1lo IL-7Rlo CXCR3lo CD62Llo; and No. 4, KLRG1lo IL-7Rhi CXCR3lo CD62Llo (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-7Rlhi 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 KLRG1lo IL-7Rhi CXCR3hi CD62Lhi (TTEM) cells accumulated in the memory CD8 T cell population due to enhanced survival and homeostatic proliferation. Thus, the KLRG1lo IL-7Rhi CXCR3hi CD62Lhi TTEM memory cell subset was marginally higher than the TCM (Supplemental Fig. 3).

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

**FIGURE 1.** Increased T-bet expression is associated with 1˚ and 2˚ effector and memory CD8 T cells with terminally differentiated phenotypes. Naive or memory P14 CD8 T cells (1 × 10^6) were transferred into naive recipients that were subsequently infected with LCMV (1˚ infection) or LM-33 (2˚ infection). A, 1˚ and 2˚ effector (day 7–8 p.i.) and memory (day 80+ p.i.) Thy1.1+ CD8+ cells were analyzed for KLRG1, IL-7R, CXCR3, CD62L, and T-bet and combodermin (Eomes) expression by flow cytometry (as shown in Supplemental Figs. 1, 2), and then the most prevalent subsets were ranked based on T-bet median fluorescence intensity (MFI) as shown in the overlaid histogram. For each subgroup, the frequency within Thy1.1+ P14 CD8+ T cells, and the MFI of T-bet and Eomes and the T-bet MFI/Eomes MFI ratio are indicated in the charts. BD ("below detection") indicates population ~1%. B, Pie charts show the frequency of different subsets in A within 1˚ or 2˚ effector (days 8 or 7), “early” memory (1–2 mo after 1˚ or 2˚ infection), and “late” memory (∼4–9 mo after 1˚ or 2˚ infection) CD8 T cells. Note the numbers of cells in each subgroup is shown in Supplemental Fig. 2. C and D, Histograms show expression of T-bet and Eomes in 1˚ (shaded) or 2˚ (line) P14 effector CD8 T cells (C) or IL-2 and GzB in 1˚ or 2˚ memory P14 CD8 cells (D). Data are representative of five to seven experiments.
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 CD27hi 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+/− 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 CD27hi CXCR3hi CD62Llo phenotype, whereas in contrast, a substantial number of Tbx21+/− 2° effector and memory CD8 T cells retained a KLRG1lo IL-7Rhi CD27lo CXCR3lo 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

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**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 × 10⁶ 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 (×10⁶) of P14 1°, 2°, and 3° effector and memory CD8+ T cells at 7 and 50+ d, respectively. Data are representative of five to seven experiments.
phenotypes during 1° and 2° infection, we tested whether a third round of infection with recombinant vaccinia virus that expresses GP$_{33-41}$ (VV-33) would push the Tbx21$^{lo}$ 2° memory CD8 T cells to upregulate T-bet and further differentiate. This showed that the Tbx21$^{lo}$ 3° effector CD8 T cells acquired more KLRG1$^{hi}$ IL-7R$^{lo}$ CD27$^{ho}$ CXCR3$^{lo}$ CD62L$^{lo}$ cells than in the prior two infections, but the frequency remained considerably smaller than the Wt 3° effector cells (Fig. 2A, Supplemental Fig. 4D). Furthermore, the Tbx21$^{lo}$ 3° effector CD8 T cells continued to express more combs and less T-bet than the Wt cells (Fig. 2B). The clonal burst of the Tbx21$^{lo}$ 3° effector 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$^{lo}$ 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 a KLRG1$^{hi}$ IL-7R$^{lo}$ CD27$^{lo}$ CXCR3$^{lo}$ 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 $\times$ 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 KLRG1$^{hi}$ IL-7R$^{lo}$ CD27$^{lo}$ CXCR3$^{lo}$ 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 GP$_{33-41}$-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 $\times$ 10$^3$ GP$_{33-41}$-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 $\sim$1.5 $\times$ 10$^5$ to 5 $\times$ 10$^5$ GP$_{33-41}$-specific CD8 T cells. This created two groups of mice in which the numbers of GP$_{33-41}$-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 $\times$ 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.
The number of preexisting memory CD8 T cells influences the differentiation state of 2° effector CD8 T cells. A–D, 5 × 10^5 unlabeled (A, B) or 10^3, 10^4, and 10^6 CFSE-labeled (C, D) Thy.1.1 P14 memory CD8 T cells from day 50+ p.i. were transferred into naive or LCMV-immune recipients (Thy.1.2+) that were subsequently infected with LM-33. E and F, LCMV-immune mice containing a range of P14 memory CD8 T cells (between ~2 × 10^5 to 5 × 10^6 and ~1.5 × 10^6 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.

FIGURE 4. The number of preexisting memory CD8 T cells influences the differentiation state of 2° effector CD8 T cells. A–D, 5 × 10^5 unlabeled (A, B) or 10^3, 10^4, and 10^6 CFSE-labeled (C, D) Thy.1.1 P14 memory CD8 T cells from day 50+ p.i. were transferred into naive or LCMV-immune recipients (Thy.1.2+) that were subsequently infected with LM-33. E and F, LCMV-immune mice containing a range of P14 memory CD8 T cells (between ~2 × 10^5 to 5 × 10^6 and ~1.5 × 10^6 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.

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 LM-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. 5 A, 5B). However, these differences were somewhat normalized when LCMV-immune recipients were infected with 100-fold more LM-33 (2 × 10^6 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 × 10^5 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
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 2° 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 2° 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 2° or even 3°, 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 2° 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 2° effector CD8 T cells have been performed by adoptively transferring small numbers of memory CD8 T cells into naive recipients and rechallenging them with 2° 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 2° 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 2° memory CD8 T cells differentiate during a natural 2° infection (26). In this study, we show that the physiological 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 1° infections (10, 17, 53). Thus, for pathogens that are serially encountered throughout life, the function, phenotype, and longevity of CD8 T cell immunity may be most affected by the starting precursor frequency of the 1° memory CD8 T cell pool and the rate at which 2° infections 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 reinfecion. Notably, T-bet concentrations did not accumulate to Wt levels in the Tbx21<sup>−/−</sup> 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 also had increased expression of eomes (Fig. 5C and data not shown), which is crucial for long-term memory cell maintenance (33, 71). Therefore, agents that limit T-bet or increase eomes 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<sup>+</sup> T<sub>EM</sub> 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 T<sub>CM</sub> also have greater telomerase activity relative to other T<sub>EM</sub> subsets (KLRG1<sup>lo</sup> IL-7R<sup>hi</sup> CXCR3<sup>lo</sup> CD62L<sup>lo</sup> and KLRG1<sup>hi</sup> IL-7R<sup>lo</sup> CXCR3<sup>lo</sup> CD62L<sup>hi</sup>), but surprisingly, we found the KLRG1<sup>hi</sup> IL-7R<sup>lo</sup> CXCR3<sup>lo</sup> CD62L<sup>lo</sup> 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 KLRG1<sup>hi</sup> IL-7R<sup>lo</sup> CXCR3<sup>lo</sup> CD62L<sup>lo</sup> 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 2<sup>e</sup> effector CD8 T cells differentiate during rechalleng. This point may be most relevant in the setting of vaccination. If a vaccine generates fairly small numbers of memory CD8 T cells, then these cells could be particularly prone to terminal differentiation upon 2<sup>e</sup> 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 larger numbers of memory CD8 T cells while minimizing the number of those cells that are terminally differentiated in the process may lead to more efficacious vaccines.

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References