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The Longevity of Memory CD8 T Cell Responses after Repetitive Antigen Stimulations

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In experimental models in which the Ag-stimulation history of memory CD8 T cell populations was clearly defined (adoptive transfer of a known number of TCR-transgenic memory CD8 T cells), all facets of the ensuing CD8 T cell responses, including proliferative expansion, duration and extent of contraction, diversification of memory CD8 T cell transcriptomes, and life-long survival, were dependent on the number of prior Ag encounters. However, the extent to which sequential adoptive-transfer models reflect the physiological scenario in which memory CD8 T cells are generated by repetitive Ag challenges of individual hosts (no adoptive transfer involved) is not known. Direct comparison of endogenous memory CD8 T cell responses generated in repetitively infected hosts revealed that recurrent homologous boosting was required to preserve the numbers and increase the phenotypic and functional complexity of the developing memory CD8 T cell pool. Although life-long survival of the memory CD8 T cells was not impacted, phenotype (i.e., upregulation of CD62L) and function (i.e., homeostatic turnover, Ag-stimulated IL-2 production) of repeatedly stimulated memory CD8 T cells were dependent on time after last Ag encounter. Therefore, repetitive Ag challenges of individual hosts can substantially influence the numerical and functional attributes of polyclonal memory CD8 T cells, a notion with important implications for the design of future vaccination strategies aimed at increasing the number of protective memory CD8 T cells. The Journal of Immunology, 2014, 192: 000–000.

Memory CD8 T cells have evolved to provide protection from repetitive infections with the same or related pathogens (1–5). The ability of functional memory CD8 T cells to directly recognize and destroy Ag-expressing infected cells represents an important component of vaccine-induced immunity against infections. Following an acute infection or vaccination, primary (1˚) memory CD8 T cells are generated from a low number of naive Ag-specific precursors that undergo a vigorous expansion phase (10,000–100,000-fold increase in numbers) that is followed by a substantial contraction phase (loss of 95% of effector CD8 T cells) (6–10). The superior protective capacity of memory CD8 T cells is linked to their increased abundance, in both lymphoid and nonlymphoid tissues, and their heightened ability to recall effector functions (cytokine production and cytolyis) relative to their naive counterparts (2, 3, 11, 12).

Memory CD8 T cells with a history of repeated Ag stimulations are generated in humans after recurring, chronic, and latent infections (13–16). Many vaccines in use today involve an initial immunization that is followed by one or more booster immunizations (prime-boost protocols) (17, 18). A recently successful clinical trial in humans used a regimen of five successive i.v. immunizations with an attenuated sporozoite vaccine to generate protection from malaria challenge, suggesting that repetitive booster immunizations were needed to generate adequate levels of pathogen-specific CD8 T cells to achieve measurable protection (19).

The impact of repeated Ag exposure on memory CD8 T cell differentiation was explored recently in well-defined murine models of infections. We (20–24) and other investigators (25, 26) showed that the number of Ag exposures dictates important phenotypic and functional characteristics of the resulting memory CD8 T cell populations. The magnitude of the proliferative expansion, duration and extent of contraction, and tissue distribution of ensuing memory CD8 T cell populations were clearly dependent on the Ag-exposure history (20, 23, 25). Importantly, every additional Ag stimulation (1˚ to quaternary [4˚]) leads to an increase in the number of differentially regulated genes and, thus, to further differentiation of memory CD8 T cells (25). As a consequence of this stepwise differentiation process, each additional Ag stimulation results in memory CD8 T cell populations that possess a unique repertoire of regulated genes and biological pathways (23).

The analysis of memory CD8 T cell populations after multiple Ag stimulations required an approach for the detection and isolation of highly pure populations with a defined number of Ag encounters. To achieve that, an adoptive-transfer system of a known number of memory CD8 T cells with fixed TCR (T cell–transgenic [Tg] CD8 T cells) was used (23). The adoptive transfer of a relatively low number of memory CD8 T cells (2–5 × 10^3 cells/mouse) ensured that every memory CD8 T cell analyzed was recruited into the response upon every subsequent infection. Although invaluable to precisely define the role of multiple Ag encounters on the development and maintenance of memory CD8 T cell populations, the extent to which a sequential adoptive-transfer model reflects the physiological scenario in which endogenous (polyclonal) memory CD8 T cells are generated by repetitive Ag challenges of individual hosts is not known.
Materials and Methods

Mice and bacteria

C57BL/6 (B6; Thy1.2; CD45.2) mice were obtained from the National Cancer Institute. TCR-Tg OT-I Thy1.1 (27), B6 Thy1.1, and CD45.1 mice were bred and maintained in the animal facilities at the University of Iowa at the appropriate biosafety levels. All mice were used at 6–8 wk of age. Attenuated acTα-deficient Listeria monocytogenes expressing OVA (Att LM-OVA) and virulent L. monocytogenes expressing OVA (Vir LM-OVA) were grown, injected i.v., and quantified as described (20, 28, 29). All animal studies were approved by the University of Iowa Institutional Animal Care and Use Committee and met stipulations of the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

Abs, peptides, tetramers, and BrdU proliferation assay

The following mAbs from eBioscience were used with the indicated specificity and appropriate combinations of fluorochromes: Thy1.1 (clones OX-7 or HIS51), CD45.2 (104), CD62L (MEL-14), CD8 (53-6.7), CD127 (A7R34), CD27 (LG.779), CD122 (SH4), IFN-γ (XMG1.2), TNF (MP6-XT22), IL-2 (JES6-5H4), and appropriate isotype controls. Synthetic OVA257–264 peptide was used as previously described (20). For analysis of BrdU incorporation, mice were injected i.p. with 2 mg BrdU (Sigma) at the beginning of the time period studied and were given drinking water containing 0.8 mg/ml BrdU. Fourteen days later, CD8 T cell proliferation was assessed in peripheral blood and tissues using an FITC BrdU Flow Kit (BD). MHC class I tetramers (Kb) specific for OVA257–264 epitope were prepared and used as previously described (17).

Adoptive-transfer experiments and isolation of lymphocytes from tissues

For adoptive transfer of naive TCR–Tg cells, 500 Thy1.1 OT-I T cells were injected i.v. into naive Thy1.2 B6 mice (30). OT-I cells were obtained from peripheral blood samples of a 2–3-mo-old donor. Contaminating memory phenotype (CD44hiCD11ahiVa2+Vb5+) OT-I cells were <5%. To transfer memory OT-I or endogenous KbOVA+ CD8 T cells (obtained after single or multiple infections of naive Thy1.1 or Thy1.2 B6 mice), we euthanized immune mice containing memory CD8 T cells at indicated time points postinfection. The percentage of memory CD8 T cells in total spleen cells was determined by FACS, and a spleen cell mixture containing 3–5 × 10^6 memory cells was injected into naive recipients 1 d before infection with L. monocytogenes expressing OVA (LM-OVA). For isolation of lymphocytes from tissues, samples of blood were obtained by retro-orbital puncture before tissue removal. Anesthetized mice were then perfused through the left ventricle with PBS. Single-cell suspensions from lung, spleen, and lymph nodes were washed before Ab staining (30).

Quantification of CD8 T cell responses and intracellular cytokine staining

OT-I T cell responses in peripheral blood and tissues were monitored by FACS analysis for Thy1.1 CD8 T cells, Endogenous KbOVA+ CD8 T cells were detected by allophycocyanin-conjugated tetramer complexes, as described (17). The percentage of CD8 T cells producing cytokines after stimulation with OVA257–264 peptide was determined using intracellular cytokine staining for IFN-γ and TNF or IL-2 after 5 h of incubation in brefeldin A (BD) (31).

Statistical analysis

Data were analyzed with Prism4 GraphPad software, and statistical significance was assessed using the two-tailed t test with a confidence interval >95%. Data in scatter dot plots are presented as mean, and data in bar graphs are presented as mean ± SD.

Results

Expansion and survival of TCR-Tg memory CD8 T cell populations after repetitive Ag stimulations

Memory CD8 T cells generated after one or multiple Ag encounters differ in their phenotype and function (20, 23, 25, 32). Importantly, the capacity of memory CD8 T cells to respond to homeostatic cytokines and undergo proliferative turnover in vivo is decreased with every additional Ag stimulation, suggesting that longevity of the memory CD8 T cell pool might be impaired as a result of the number of prior Ag encounters (23, 25). To determine whether long-term survival of memory CD8 T cell populations is impaired in multiply stimulated memory CD8 T cells, low and physiological numbers of naive TCR–Tg Thy1.1 OT-I CD8 T cells (5 × 10^4/mouse) (30, 33) were adoptively transferred into naive Thy1.2 B6 hosts before challenge with Att LM-OVA. At memory time points (>2 mo postinfection), 1° memory OT-I CD8 T cells (5 × 10^7/mouse) from the spleen were again adoptively transferred, whereas another group of recipient mice received naive OT-I CD8 T cells before the Att LM-OVA infection. Adoptive transfers were repeated until 1° to quinary (5°) memory CD8 T cell populations could be studied simultaneously (Fig. 1A). The use of relatively small numbers of adoptively transferred naive and memory CD8 T cells ensured that all Thy1.1 OT-I cells detected had recognized the cognate Ag and expanded in numbers in response to every Att LM-OVA challenge.

In the experiment shown in Fig. 1, the same numbers of 1°, 4°, and 5° memory OT-I CD8 T cells were transferred into groups of naive Thy1.2 B6 recipients prior to Att LM-OVA infection. Consistent with our previous report using 1°, secondary (2°), and tertiary (3°) memory CD8 T cell populations (23), the stimulation history of input memory cell populations had a clear influence on the effector CD8 T cell accumulation in vivo (Fig. 1B). When compared on a per-cell basis, the proliferative expansion in the numbers of 5° memory CD8 T cells was two orders of magnitude lower than observed with 1° memory CD8 T cells (Fig. 1B). However, it is possible that, upon adoptive transfer, uptake (seeding) and survival of memory CD8 T cells differ between 1° and multiple-stimulated memory CD8 T cells, influencing the number of memory CD8 T cells available to respond to additional Ag stimulation. To address this, Thy1 disparate 1° and 3° memory CD8 T cells were cotransferred into naive B6 hosts, and their distribution was determined in various organs 1 d after adoptive transfer. As described previously (23), consistent with their phenotype (i.e., lower frequency of CD62L-expressing cells), the accumulation of repeatedly stimulated memory CD8 T cells is decreased in lymph nodes compared with 1° memory CD8 T cells. However, equal, if not increased, numbers of 3° memory CD8 T cells were observed in all other organs examined, including spleen, which represents a major organ where pathogen recognition and activation of memory CD8 T cells occur after L. monocytogenes infection (34) (Supplemental Fig. 1). Similarly to the data shown in Fig. 1B, the proliferative expansion of 1° memory CD8 T cells was significantly higher than was the expansion of 3° memory CD8 T cells upon L. monocytogenes infection (data not shown). Thus, these data confirm that each Ag stimulation further erodes the ability of memory CD8 T cell populations to proliferate and/or accumulate in vivo.

Importantly, this experimental setup enabled longitudinal analysis of CD8 T cell responses in individual mice to determine whether long-term survival of memory CD8 T cell populations is impaired by the Ag-stimulation history. As shown previously, the numbers of 1° and 2° memory CD8 T cells can stabilize after the resolution of the contraction (death) phase, and those populations can be maintained for the life of the host (Fig. 1C) (20, 23, 25, 32). However, the long-term survival was compromised in mice that contained 5° and senary (6°) CD8 T cells. At >200 d postchallenge, CD8 T cells stimulated six times were undetectable in the PBLs (Fig. 1C, 1D) and all other organs examined (spleen, lung, lymph nodes; data not shown). These results suggest that, under the experimental conditions used in this study (series of adoptive transfer and vigorous proliferative expansion of small numbers of monoclonal TCR
memory CD8 T cell populations after each restimulation), persistence and survival of memory CD8 T cells are influenced by the number of Ag exposures.

Multiple Ag encounters impair maintenance, function, and tissue distribution of endogenous memory CD8 T cell populations in an adoptive-transfer model

To test the extent to which the results obtained with monoclonal TCR-Tg CD8 T cells are observed with endogenous (polyclonal) CD8 T cells, naive Thy1.1 B6 mice were infected with Att LM-OVA (Fig. 2A). At the memory time point (>2 mo postinfection), splenocytes that contained $1 \times 10^5$ KbOV A257-specific CD8 T cells were adoptively transferred into naive Thy1.2 B6 recipients before 2° infection. These transfers were repeated until 4° CD8 T cell responses could be studied (Fig. 2A). Similar to the data obtained with TCR-Tg CD8 T cells, the number of Ag encounters dramatically influences the differentiation, maintenance, and function of endogenous memory CD8 T cell populations. The long-term survival was impaired (Fig. 2B–D), and the frequency of memory CD8 T cells expressing phenotypic/functional markers known to be involved in CD8 T cell memory differentiation (CD127, CD62L, CD27hi) (35–37) (Fig. 2E) was decreased in repetitively stimulated memory CD8 T cell populations. In addition, the ability to undergo homeostatic turnover (as determined by BrdU incorporation) was greatly reduced in 4° memory CD8 T cells compared with 1° memory CD8 T cells of the same specificity (Supplemental Fig. 2A–C). Consistent with their CD62Llo phenotype (Supplemental Fig. 2D), repeatedly stimulated CD8 T cells were excluded from lymph nodes and preferentially accumulated in 3° tissues, such as lungs (Supplemental Fig. 2E). Thus, these data show that maintenance, function, and tissue distribution of polyclonal CD8 T cell responses generated in a sequential adoptive-transfer model are influenced by the Ag-stimulation history.

Endogenous memory CD8 T cell responses after repetitive Ag stimulation of individual hosts

Protection from infection directly correlates with the number of existing memory CD8 T cells, and multiple Ag stimulations (prime-boost protocols) represent a viable approach to increase the quantity of memory CD8 T cells (1). However, the results that we obtained using sequential adoptive transfer of a relatively small number of TCR-Tg or polyclonal memory CD8 T cells showed that the number of Ag encounters can decrease the functionality and maintenance, as well as change the phenotypic composition, of the memory CD8 T cell pool (Figs. 1, 2). If taken to the extreme, these data also suggest that longevity and/or function of memory CD8 T cell responses in any individual might be compromised with repetitive Ag stimulation.

Therefore, to test the extent to which repetitive Ag stimulation of individual hosts (no adoptive transfer involved) controls the ensuing memory CD8 T cell responses, naive B6 mice were infected with Att LM-OVA (Fig. 3A). Forty days after 1° infection, immune mice were boosted (2° challenge) with $\sim$10 LD50 Vir LM-OVA (a lethal dose for naive B6 mice that is cleared from L. monocytogenes–immune mice in $\leq 48$ h; data not shown), and the same dose/challenge was repeated every 40–60 d for a total of six stimulations (Fig. 3A). The numbers of endogenous KbOVA257-specific memory CD8 T cell responses in the blood of the individual mice were determined before every challenge. Secondary challenge significantly increased the number of memory CD8 T cells (2-fold) compared with the numbers achieved with a single immunization (Fig. 3B–D). Interestingly, this longitudinal analysis of individual mice showed that additional stimulations (3° to 6°) did not increase the frequency of OVA257-specific memory CD8 T cells achieved after 2° stimulation (Fig. 3B–D). Because the magnitude of proliferative expansion and clearance of the infection are dependent on the number of memory CD8 T cells present (6), it is possible that the constant dose of L. mono-
cytogenes used for all booster challenges was not able to activate (recruit) all of the memory CD8 T cells in vivo. The recruitment of the OVA257-specific CD8 T cells were adoptively transferred into naive Thy1.2 hosts before 2° Att LM-OVA infection. The infections and adoptive transfers were repeated until 3° memory CD8 T cells were stimulated for the fourth time. (B) Endogenous K°OVA+ Thy1.1 CD8 T cells were analyzed in PBLs of individual mice at indicated days postinfection. Numbers represent the frequency of K°OVA+ T cells in total PBLs from one representative mouse analyzed longitudinally throughout the experiment. (C) Kinetics of K°OVA+ T cell responses in PBLs of individual mice at indicated days after 4° infection. Each line represents an individual mouse. (D) Percentages of K°OVA+ T cells in PBLs at indicated days were normalized to the percentage of K°OVA+ T cells at day 36 for each individual mouse. The numbers represent p values that were determined by paired, two-tailed t test. (E) Phenotype (CD127, CD62Lhi, and CD27hi) of 4° K°OVA+ (upper panels) and K°OVA− (lower panels) CD8 T cells in PBLs 90 d postinfection. Open graphs represent isotype-control staining. Numbers represent the frequency of CD8 T cells positive for the indicated markers. One of two experiments with similar results is shown. ns, not significant.

Continuous boosting of Ag-specific CD8 T cells preserves the numbers and increases the complexity of memory CD8 T cell populations

Tertiary and every subsequent challenge in individual hosts did not lead to a further increase in the numbers of memory CD8 T cells achieved after 2° infection, suggesting that the memory cell numbers were not dependent on the number of times that pathogen was introduced (Fig. 3). Alternatively, continuous boosting may be required to preserve the numbers of memory CD8 T cells, because repetitively stimulated CD8 T cell responses are programmed to undergo a vigorous and protracted contraction phase (6, 23–25, 39). To address these possibilities, additional experiments were performed in which one group of mice was infected three times; polyclonal K°OVA257-specific CD8 T cell responses were analyzed and compared with mice that were continuously challenged a total of six times (Fig. 4). Interestingly, despite the inability of the booster challenges to increase memory CD8 T cell numbers over the levels achieved before the last Ag encounter, continuous boosting is required to preserve the numbers of OVA-specific CD8 T cells (Fig. 4A). Interestingly, phenotypic analysis performed 9 mo after initiation of the experiment showed that the subset composition of OVA257-specific CD8 T cell responses differs between hosts that were stimulated three or six times. In the mice that were infected three times, the percentage of OVA257-specific CD8 T cells expressing the surface molecules usually associated with “long-term” memory CD8 T cell differentiation (i.e., CD127, CD62L, CD27hi) (35–37) was increased. However, because of the -6-fold increase in the overall numbers of memory CD8 T cells detected in hosts stimulated six times, the number of memory CD8 T cells that expressed high levels of CD62L was similar, if not increased, compared with the mice that received only three challenges (difference in the frequency of CD62Lhi CD8
T cells was ∼3.3-fold) (Fig. 4B). Therefore, continuous boosting is required to preserve the numbers and increase the subset heterogeneity of memory CD8 T cell populations that have been subjected to multiple prior Ag stimulations.

Longevity of endogenous memory CD8 T cells after repetitive Ag stimulation of individual hosts

To explore the extent to which life-long survival of endogenous memory CD8 T cell responses was influenced by the Ag-stimulation history, extended longitudinal analysis was performed in groups of mice described in Fig. 4. Interestingly, ∼200 d after the last (6˚) infection, the frequency of OVA257-specific CD8 T cells in the blood (Fig. 5A) and the frequency of the memory CD8 T cells expressing high levels of CD62L (Fig. 5B) were similar in the groups of mice that were challenged three or six times. In addition to the PBL analysis, the total number, function, and distribution of OVA257-specific CD8 T cells were determined in various organs 450 d after the initiation of the experiment. Total numbers of OVA257-specific memory CD8 T cells in the spleen (Fig. 5C) and their ability to undergo homeostatic proliferation (Fig. 5D) were indistinguishable between groups. The distribution of memory CD8 T cells in hosts stimulated three or six times was similar in 2˚ lymphoid organs and 3˚ tissues analyzed (Fig. 6A), and both memory CD8 T cell populations efficiently produced IFN-γ in re-

FIGURE 3. Repetitive Ag stimulation of individual hosts does not lead to the loss of endogenous CD8 T cell responses. (A) Experimental design. Naive B6 mice were infected with Att LM-OVA (5 × 10^6 CFU; i.v.) and subsequently reinfected every 40+ d with Vir LM-OVA (1 × 10^7 CFU; i.v.). (B) The frequency of tetramer-positive K\textsuperscript{b}OVA\textsuperscript{+} memory CD8 T cells in the blood of individual mice 40+ d after the last LM-OVA challenge. Numbers represent the percentage of K\textsuperscript{b}OVA\textsuperscript{+} CD8 T cells in total PBLs from a representative mouse. (C) Frequency of K\textsuperscript{b}OVA\textsuperscript{+} memory CD8 T cell responses in PBLs at day 40+ after last challenge. (D) Fold change in the frequency of K\textsuperscript{b}OVA\textsuperscript{+} CD8 T cells between subsequent challenges. Data are mean ± SD for five mice/group and are representative of three similar experiments.

FIGURE 4. Continuous boosting of Ag-specific CD8 T cells is required to preserve the numbers and increase the complexity of memory CD8 T cell populations. Groups of B6 mice were infected with Att LM-OVA (1˚ infection) and later challenged with Vir LM-OVA two additional times (3˚ infection group) or five additional times (6˚ infection group). (A) Kinetics of K\textsuperscript{b}OVA\textsuperscript{+} CD8 T cell responses in PBLs of 3˚ and 6˚ challenged mice were analyzed on the indicated days. Data are mean ± SD for five mice/group. Vertical dashed lines indicate the time of infection. (B) Phenotype of K\textsuperscript{b}OVA\textsuperscript{+} CD8 T cells in 3˚ and 6˚ challenged mice at day 271 from the start of the experiment. Numbers represent the frequency of K\textsuperscript{b}OVA\textsuperscript{+} CD8 T cells positive for CD127, CD62L\textsuperscript{hi}, or CD27\textsuperscript{hi}. One of two experiments with similar results is shown.
response to in vitro peptide stimulation (Fig. 6B–D). Finally, similar frequencies of IFN-γ–producing memory CD8 T cells were able to coproduce TNF (Fig. 6E, 6F) and IL-2 (Fig. 6E, 6G) directly ex vivo. To prove that memory CD8 T cells can survive for the life of the repetitively stimulated hosts, endogenous OVA257-specific CD8 T cell responses were analyzed 21 mo after the initiation of the experiment. Data are mean ± SD for four or five mice/group. (B) CD62L expression on Kb OVA+ memory T cells at day 442 postinfection. Numbers represent the frequency of KbOVA+–specific CD8 T cells that regain expression of CD62L molecule. (C) Total numbers of KbOVA+ memory CD8 T cells in the spleens (day 452 postinfection) of individual mice. (D) Frequency of BrdU+KbOVA+ memory CD8 T cells in the spleens of individual mice. Horizontal lines in (C) and (D) represent the mean. One of two experiments with similar results is shown.

Discussion
The protective capacity of memory CD8 T cell populations in vivo is dependent on their abundance and phenotype (subsets compo-
positive for the indicated markers. Phenotype of KbOVA+ CD8 T cells from the spleens of 3˚ and 6˚ challenged mice at day 629 postinfection. Numbers represent the frequency of KbOVA+ CD8 T cells positive for the indicated markers.

Figure 7. Survival of endogenous memory CD8 T cells from repetitively infected hosts is not influenced by Ag-stimulation history. (A) The frequency of KbOVA+ memory CD8 T cell responses in PBLs at day 629 post-1˚ infection. (B) Phenotype of KbOVA+ CD8 T cells from the spleens of 3˚ and 6˚ challenged mice at day 629 postinfection.

Recent studies also suggest that an extremely large threshold in memory CD8 T cell frequencies is required for achieving long-term protection against specific pathogens (15, 40). Therefore, multiple Ag stimulation of individual hosts represents a valid approach to increase the numbers of memory CD8 T cells. How Ag-stimulation history influences the survival of polyclonal (endogenous) memory CD8 T cells and the extent to which the findings observed in adoptive-transfer models are translatable to the CD8 T cell responses that are generated in individual hosts has not been directly compared. In this study, in direct analysis of endogenous memory CD8 T cell responses generated in individual hosts that were reinfeected a different number of times, we showed that recurrent boosting did not lead to disappearance of memory CD8 T cells, a phenomenon only observed in adoptive-transfer models in which the Ag-stimulation history of memory CD8 T cell populations was clearly defined and the contribution of new naive CD8 T cell precursors was excluded, phenotype, function, tissue distribution, and molecular signatures of memory CD8 T cell populations were controlled by the number of Ag encounters (23).

We extended these studies to show the impact of repetitive Ag stimulations on the ability of the host to maintain its TCR-Tg or polyclonal memory CD8 T cell pool. At 7 mo postinfection, we observed the loss of memory in 30% of mice that hosted 5˚ memory CD8 T cells and in 100% of mice that hosted 6˚ memory CD8 T cells. Thus, survival of the repetitively stimulated TCR-Tg memory CD8 T cell pool was clearly dependent on the number of times that those cells had responded to cognate Ag.

How many times a memory CD8 T cell has to see an Ag/infected to show functional impairments or lose the ability to survive in vivo is likely to be variable and dependent on multiple factors. To address how Ag-stimulation history influences the long-term maintenance of the resulting memory CD8 T cell pool, the numbers of memory CD8 T cell used in sequential adoptive transfers, the age (defined as time after the last Ag encounter) of the transferred memory CD8 T cells, and the dose of the infection were kept constant. As in adoptive-transfer experiments involving TCR-Tg CD8 T cells, the potential permutations in the experimental design using serial infection of individual mice are numerous and should be taken into account. The dose and type (homologous or heterologous) of booster immunization and time between challenges can potentially influence the generation, differentiation, and maintenance of multiply stimulated memory CD8 T cell pools in individual hosts. We would argue that the adoptive-transfer model that enables direct comparison of various CD8 T cell populations could represent a viable approach to precisely define other variables (such as number, age, subsets of memory used in sequential adoptive transfers, dose type of the infection/immunization) that might play a role in the generation and maintenance of memory CD8 T cell responses.

An important variable that has to be taken into consideration is the age of the memory CD8 T cells compared in these studies. It was shown that the decrease in naive CD8 T cell precursor frequency, as well as qualitative changes in CD8 T cells during aging (upregulation of CD44 and inhibitory receptors, including PD-1 and LAG3), is related to impaired CD8 T cell–mediated immunity to infection (42). It is less clear whether CD8 T cell memory established early in life also becomes impaired with age (43). Future studies will be designed to determine the extent to which aging controls differences observed between 1˚ and repetitively stimulated memory CD8 T cells, although those studies will be complicated by the fact that memory CD8 T cells undergo constant phenotypic and functional changes, even in the absence of further Ag encounter (Figs. 4, 7) (M.D. Martin and V.P. Badovinac, manuscript in preparation). It is tempting to speculate the extent to which these differences in functionality of memory CD8 T cells could be magnified in humans that have a longer life span and the potential to re-encounter Ag multiple times (13–15).

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Disclosures
The authors have no financial conflicts of interest.

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