Defective CD8 T Cell Responses in Aged Mice Are Due to Quantitative and Qualitative Changes in Virus-Specific Precursors

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Aging is associated with a decline in T cell function and an increase in susceptibility to infections. Some of the immunological changes associated with aging include reduced thymic output, a decrease in naive T cells, expansion of memory phenotype CD8 T cells, cellular senescence, changes in telomerase expression or telomere length, reduced proliferation, altered signaling, and reduced IL-2 production (1–5). Two prominent age-associated changes, the involution of the thymus and a global shift of T cell phenotype from naive to memory, lead to a decrease in the number of naive T cells (CD44lo) and an increase in the proportion of T cells with memory phenotype (CD44hi) (6, 7). Several studies showed that the transfer of CD44lo CD8 T cells into Rag-1−/− recipients (lymphopenic environment) results in the transition to a CD44hi memory phenotype within 3 wk. Using adoptive transfer of different numbers of naive (CD44lo) cells to syngeneic Rag-1−/− recipients, the transition to a memory (CD44hi) phenotype was demonstrated to be directly proportional to the extent of homeostatic proliferation (8), suggesting that the gradual decline in new naive T cell production during aging could lead to a gradual increase in homeostatic proliferation of the existing T cell pool. At least in young mice, T cells generated during lymphopenia-induced homeostatic proliferation have increased functionality after in vivo Ag stimulation compared with naive CD44lo T cells (9), and these homeostatically induced CD44hi T cells provided protective immunity to bacterial infections (10). However, two subpopulations of proliferating T cells exist under lymphopenic conditions, a slow proliferative subset, and a subset that rapidly proliferates to CFSE negative (11). Interestingly, the rapidly proliferative subset is thought to be driven by environmental and/or self-Ags and upregulates the inhibitory receptor programmed death-1 (PD1) (12). It is unclear, however, if changes in the T cell compartment prior to infection, whether driven by lymphopenia or other signals, impact antiviral immunity.

A diverse repertoire of naive T cells is essential for a vigorous response to infection (13, 14). Reduced thymic output can result in a decline in naive Ag-specific precursors, and this decline can impact the generation of an effective immune response (15, 16). Moreover, clonal expansions of memory phenotype T cells have been observed in mice, primates, and humans (17). These expansions are believed to be driven by chronic infections, altered cytokine milieu of the host, or both and could further restrict the T cell repertoire and/or compete with other T cell responses (18). Thus, changes in the global T cell pool and/or changes in T cell competition might account for some changes in T cell immunity observed with age.

Although less well documented, cell-intrinsic changes of naive T cells could also contribute to impaired immunity with aging. Recently, several studies observed phenotypic alterations in the CD4 T cell pool in aged mice including changes in the expression of several molecules such as PD1, ICOS, CTLA4, and KLRG1, although the consequences of these phenotypic changes remain poorly understood (19–21). Recent studies also demonstrated that decreased thymic output with age leads to an accumulation of long-lived naive CD4 T cells that express low amounts of Bim and have functional defects (22). Less information is available regarding the impact of aging on the naive CD8 T cell pool.
In this study, we demonstrated that aging in mice was associated with an increase in the expression of several inhibitory receptors including PD1, Lag3, 2B4, and CD160 on CD8 T cells. These changes were especially pronounced on the CD44 Hi population of CD8 T cells from uninfected aged mice with changes first becoming apparent sometime between 8 and 12 mo of age concurrent with a shift from CD44 Lo to predominantly CD44 Hi T cells. We also demonstrated that the precursor frequency of virus-specific CD8 T cells from uninfected aged mice declines by ±10-fold. Moreover, aging resulted not just in a decreased precursor frequency but also in changes in the quality of these precursors. Many Ag-specific CD8 T cell precursors were CD44 Hi and PD1 positive. Transcriptional profiling of total CD44 Lo and CD8 T cells from naive aged mice revealed strong similarities to exhausted CD8 T cells found during chronic infections. Studies with aged and young TCR transgenic T cells indicated substantial proliferative defects of CD44 Hi T cells from uninfected mice. These studies suggest both quantitative and qualitative defects in the pool of Ag-specific CD8 T cells prior to immunization or infection in the elderly and point to possible opportunities for reversing such age-related defects.

Materials and Methods

Mice

Female mice were purchased from Taconic at either 3–5 or 8 mo of age and then aged to 12–14 and 17–22 mo old (aged mice) in the Wistar Institute aging colony. Additional male mice were purchased from Taconic at 2–3 mo of age. These mice were housed in the same colony but used when 3–5 mo old (young mice). Animals were housed in microisolator cages at the American Association for the Accreditation of Laboratory Animal Care-approved Animal Care Facility at the Wistar Institute and screened by an independent commercial service (Charles River Laboratories) every 6 mo for a panel of 17 rodent pathogens including LCMV and mouse norovirus (MNV). All screens for rodent pathogens over the 5 y of existence of this aging colony were negative. In an independent set of studies, aged mice (17–22 mo old) were obtained from the aging colony at the National Institutes on Aging and examined directly upon receipt. Aged animals with obvious cancer or lymphoma were excluded from the studies. For all adoptive transfer experiments, congenic mice (obtained from National Cancer Institute) differing in Ly5.1 versus Ly5.2 or Thy1.1 versus Thy1.2 were used. P14 TCR transgenic mice were bred at the Wistar Institute. Ot1 Rag2−/− female mice were obtained from Taconic at 2 mo of age. All mice were used in accordance with Institutional Animal Care and Use Committee guidelines.

Adoptive transfer, virus, and infection

CD8 T cells from young and aged P14 Ly5.1 or P14 Thy1.1 mice were purified (>90% purity) using magnetic beads (CD8 T cell isolation kit, MACS beads; Miltenyi Biotec) and then sorted on the basis of CD44 expression. After sorting, 5 × 10^5 P14 (D^GP33,-specific) CD8 T cells from each group (young CD44Lo, young CD44Hi, aged CD44Lo, and aged CD44Hi) were transferred i.v. into wild-type recipient mice (Ly5.2+ Thy1.2−), which were then infected with LCMV Armstrong (2 × 10^6 PFU) i.p. Donor populations were monitored in the peripheral blood by retro-orbital blood collection as described previously (23). Similarly, CD8 T cells from young OT1 Rag2−/− mice were purified using magnetic beads (CD8 T cell isolation kit, MACS beads; Miltenyi Biotec) and then sorted on the basis of CD44 expression. After sorting, 15 × 10^5 OT1 (K^OVA specific) CD8 T cells from each group (young CD44Lo and young CD44Hi) were transferred i.v. into wild-type recipient mice (Ly5.2−). These were then infected with recombinant vesicular stomatitis virus expressing OVA (2 × 10^6 PFU) i.v. Donor populations were monitored in the peripheral blood by retro-orbital blood collection as described previously (23).

Isolation of lymphocytes from tissues

Lymphocytes were isolated from peripheral blood and spleens as described previously (24).

Tetramer staining and magnetic bead sorting

Spleens were isolated from uninfected mice at 4, 7, 12, 14, 17, and 19 mo of age; tetramer staining (D^GP33 or K^OVA) and magnetic bead sorting were done as described previously (9, 25). CD8 and CD3 were used as positive gates. B220, CD19, CD4, CD11b, CD11c, and MHC II were used as dump gates. In addition to tetramer staining, samples were stained for CD44 and PD1.

Flow cytometry, intracellular cytokine staining, and BrdU staining

Lymphocytes were stained using standard techniques and analyzed by flow cytometry. Virus-specific CD8 T cells were quantified using MHC class I peptide tetramer staining. MHC class I peptide tetramers were made and used as described previously (23). Abs to CD8, CD44, PD1 (RMPI-30), CD3e (145-2C11), and CD28 (37.51) were purchased from BioLegend (San Diego, CA). Abs to Lag3, 2B4, CD160, and IFN-γ were purchased from eBioscience (San Diego, CA). Abs to CD4, Mip1α, KLRG1, and Tbet were purchased from Invitrogen (Carlsbad, CA); R&D Systems (Minneapolis, MN), Beckman Coulter (Fullerton, CA), or Santa Cruz Biotechnology (Santa Cruz, CA), respectively. All other Abs were purchased from BD Biosciences (San Diego, CA). Staining and analysis were performed as described previously (23).

Function was investigated by intracellular cytokine staining following stimulation. Briefly, 1 × 10^6 splenocytes were cultured in the absence or presence of the anti-CD3 (2 µg/ml) and anti-CD28 (1 µg/ml) for 5 h at 37°C. Following staining for surface Ags as described above, cells were stained for intracellular cytokines (IFN-γ, IL-2, and Mip1α) using the Cytofix/Cytoperm kit (BD Biosciences).

For BrdU analysis, mice received a single i.p. injection (1 mg/mouse) of BrdU 12 h before sacrifice. BrdU staining was carried out using a BrdU Flow Kit (BD Pharmingen) in accordance with the manufacturer’s instructions. BrdU was detected using FITC-conjugated anti-BrdU Ab.

Samples were collected using an LSR II flow cytometer (BD Biosciences, San Jose, CA).

Cell sorting and microarray analysis

CD8 T cells from uninfected young and aged mice were purified (>90% purity) using magnetic beads (CD8+ T cell isolation kit, MACS beads; Miltenyi Biotec) and then sorted directly into TRIzol (Life Technologies, Rockville, MD) on the basis of CD44 expression. Purification was assessed by FACS analysis and was >95–98% for all samples. Total RNA was isolated from sorted CD44Hi CD8 T cells from uninfected young and aged mice using TRIzol, according to the manufacturer’s instructions. cDNA was fragmentated, labeled, and amplified as described previously (26). Samples were hybridized to the Affymetrix GeneChip Mouse Exon 1.0 ST arrays (Affymetrix, Santa Clara, CA) at the University of Pennsylvania Microarray Core Facility. Transcript levels were summarized from the Affymetrix-CEL files using the Robust Multichip Average algorithm (27), as implemented by the Affymetrix Power Tool apt-probe set summarizer. There were four technical replicates for each young and aged CD44Hi population. Differentially expressed genes were identified using the Class Neighbors module of GenePattern (28) and the Significance Analysis of Microarrays algorithm (29). Gene Set Enrichment Analysis (GSEA) was performed as described previously (26).

Statistical analysis

Data were analyzed using a two-tailed Student t test, and p ≤ 0.05 was considered significant. For microarray data analysis, we used the Class Neighbors and Significance Analysis of Microarrays algorithms as described previously (28, 29).

Results

Phenotypic changes of T cells from uninfected mice during aging

To begin to understand age-related changes in antiviral immunity, we analyzed the percentages and numbers of T cells in mice at different times in their lifespan. The analysis of mice at 4, 8, 12, and 18 mo of age demonstrated that the percentages and numbers of CD8 T cells were diminished with age. This was true in both spleen and blood (Fig. 1A, Supplemental Fig. 1A), consistent with previous studies (30–33). Moreover, we demonstrated that a previously observed global shift from naive to memory phenotype T cells (13, 34, 35) that results in an increase in the frequency and number of CD44Hi T cells and a concomitant decrease in the number of CD44Lo naive T cells started as early as 8–12 mo of age (Fig. 1A–C).
We next examined the phenotype of T cells from the spleen and blood of uninfected aged and young mice and found an increase in the expression of KLRG1 and Tbet for CD8 T cells from uninfected aged mice (Supplemental Fig. 1B, 1C), which could reflect senescence or terminal differentiation as reported previously (36, 37). Expression of other markers such as CD44, CD62L, and CD49d (α4 integrin) was also altered (data not shown), but one of the most striking observations was the difference in expression of inhibitory receptors between CD8 T cells from uninfected aged and young mice. In particular, CD44Hi CD8 T cells from aged mice had higher expression of PD1, LAG3, 2B4, and CD160 compared with CD8 T cells from young mice (Fig. 1D). The upregulation of inhibitory receptor expression started between 8 and 12 mo of age and became more prominent between 12 and 18 mo (Fig. 1E, Supplemental Fig. 1D). The change in inhibitory receptor expression with age was also evident in the total population of CD8 T cells, though most of the increased expression of PD1, LAG3, 2B4, and CD160 was restricted to the CD44Hi subset (Fig. 1D; data not shown). Although many of these inhibitory receptors are involved in self-tolerance and are upregulated by self-reactive T cells (38), these molecules also can be induced during chronic infections (39). Mice in our aging colony were routinely screened for common rodent pathogens and were always negative for all agents tested including LCMV and MNV (data not shown). Moreover, we observed a similar upregulation of inhibitory receptors on T cells from an independent source of aged mice by examining mice from the aging colony operated by the National Institutes on Aging (Supplemental Fig. 1G, 1H).

The increase in the inhibitory receptors on CD8 T cells from aged mice is not due exclusively to oligoclonal expansions

To investigate whether the increase in inhibitory receptors on CD8 T cells from aged mice was due to oligoclonal expansions, which have been associated with aging (17), we examined TCR Vβ expression on CD8 T cells from young (4 mo old) and aged (20–22 mo old) uninfected mice. Aged mice had higher variability in the expression of Vβ receptors compared with young animals in agreement with prior work (Fig. 2A, 2B) (40), and in some mice, the clonal expansions of certain Vβ receptors (e.g., Vβ4, Vβ8.3, Vβ9, Vβ13, and Vβ14) were detected. Although modest clonal expansions were occasionally present, they represented at most

FIGURE 1. Age-associated changes in CD8 T cells. Frequencies of CD8 T cells were analyzed in the spleens and blood of uninfected mice at 4, 8, 12, and 18 mo of age (A). The percentages and numbers of CD44Hi and CD44Lo CD8 T cells in the spleens of uninfected mice at age of 4–5, 8, 12, and 18 mo are shown in B and C, respectively. CD8 T cells were isolated from uninfected young and aged spleens at 4, 8, 12, and 18 mo of age and stained for the expression of inhibitory receptors PD1, LAG3, 2B4, and CD160. The representative graphs of individual stains of CD8 T cells from spleen are shown in D. The percentages of different inhibitory receptors on CD44Hi CD8 T cells from spleens are shown in E. A total of 1.48 ± 0.09% (mean ± SEM) of CD44Hi CD8 T cells in the spleens of 4-mo-old mice are PD1 positive compared with 2.58 ± 0.33, 15.93 ± 6.12, and 43.18 ± 2.81% of PD1-positive CD44Hi CD8 T cells from the spleens of 8-, 12-, and 18-mo-old mice, respectively. Data are representative of two independent experiments including 12–20 mice/experiment. Some data are representative of several pooled experiments. *0.05 > p > 0.01, **0.01 > p > 0.001, ***p < 0.001 by unpaired two-tailed t test.
Aged mice (19.5 mo) exhibited at least a 10-fold reduction in the number of D\textsuperscript{b}GP33-specific CD8 T cell precursors per spleen (~20–30 D\textsuperscript{b}GP33-specific precursors/spleen). Similarly, the number of K\textsuperscript{b}OVA-specific CD8 T cell precursors in the spleens of aged mice was significantly reduced compared with the number of these precursors in the spleens of young mice. Young mice (7 mo old) contained ~61 K\textsuperscript{b}OVA-specific precursors/spleen, whereas in aged mice (19 mo), the number of K\textsuperscript{b}OVA-specific CD8 T cell precursors per spleen was ~13 (Fig. 3E). These observations suggest that to reach the same numbers of Ag-specific T cells at the peak of the response, virus-specific CD8 T cells in aged mice would have to undergo at least three extra rounds of division, assuming Ag-specific precursors in aged mice are qualitatively similar to those in young mice. To investigate this second issue, we examined differentiation markers expressed by virus-specific precursors in uninfected aged versus young mice. In aged mice, a higher proportion of D\textsuperscript{b}GP33- or K\textsuperscript{b}OVA-specific precursors were CD44 HI (Fig. 3D, E). Thus, the numbers of Ag-specific precursors declined with age, and the Ag-specific precursors that remained were phenotypically altered.

**Functional changes of T cell populations from naive mice during aging**

We next investigated whether the changes in the phenotype and precursor frequency of the CD8 T cell pool from aged uninfected mice were accompanied by changes in the functionality of these cells. T cells from young and aged naive mice were stimulated in vitro using anti-CD3 and anti-CD28 Abs, and cytokine production was examined. During polyclonal stimulation, there were numerically more IFN-\(\gamma\) producers in aged mice (Fig. 4A, 4B). Young and aged CD8 T cells produced similar amounts of Mip1\(\alpha\), but CD8 T cells from aged mice were poor producers of IL-2 compared with CD8 T cells from young mice in agreement with previous data examining CD4 T cells from aged mice (63). We failed to detect IL-17 in the in vitro CD8 T cell assay, but we observed that aged CD8 T cells had slightly higher IL-10 production than young CD8 T cells upon activation (data not shown).

Although both aged and young CD44\textsuperscript{HI} CD8 T cells produced IFN-\(\gamma\), there were some aged mice in which this function was well
preserved and another subset of animals in which the CD8 T cells appeared less functional per cell (Fig. 4C, 4D). IFN-γ production was also compared with PD1 expression. Very little IFN-γ was found in the small PD1+ subset of CD8 T cells from young naive mice (Fig. 4E). Although there were some aged mice in which IFN-γ was produced by PD1+ CD8 T cells, in general, the PD1+ CD8 T cells from aged mice made less IFN-γ per cell compared with the PD1hi cells. Overall, these results indicate that aging alters some components of CD8 CD44hi T cell cytokine responses, although there was clearly heterogeneity with preserved functionality in some mice and perhaps moderately reduced IFN-γ production per cell in other mice.

The transcriptional profiles of CD44hi CD8 T cells from uninfected aged mice indicate a signature similar to that of exhausted CD8 T cells

We next examined the global gene-expression profiles of CD44hi CD8 T cells from young and aged mice. CD44hi CD8 T cells from young and aged uninfected mice showed differential expression of a number of transcripts known to play important roles in CD8 T cells (Fig 5A, Supplemental Tables I, II). Using both the Class Neighbors and the Significance Analysis of Microarrays algorithms, we identified many transcripts that have also been implicated in modulating CD8 T cell exhaustion or dysfunction during chronic infections (e.g., PD1, LAG3, IL-10, and Blimp-1; Supplemental Tables I, II) (26). To further probe the similarities between aging and exhaustion, we used GSEA to evaluate the global similarity between our samples and a previously defined signature of exhausted CD8 T cells (26). There was a clear enrichment of the transcriptional signature of CD8 T cell exhaustion in the aged CD44hi CD8 T cells, with a p < 0.001 (Fig. 5B). Moreover, there is a corresponding bias of a similarly defined memory signature toward the young CD44hi CD8 T cell samples, p < 0.001. Taken together, these gene expression data indicate that aging is associated with a transcriptional program in CD8 T cells that has similarities to that found in exhausted T cells. One notable set of differences, however, was the increase in KLRG1 and Tbet expression by CD8 T cells from aged mice. In contrast, expression of these molecules is reduced in exhausted CD8 T cells during chronic LCMV infection (26, 44). Nonetheless, inhibitory receptors, cell death pathways, and transcription factors were identified as specific genes and pathways and may provide important targets for future analysis.

Poor responsiveness of Ag-specific CD44hi CD8 T cells from uninfected aged and young mice

We next examined the impact of qualitative changes in the pool of Ag-specific CD8 T cells from uninfected aged mice in vivo during viral infection. Hence, we isolated DGP33-specific CD8 T cells from uninfected young and aged P14 TCR transgenic mice that are reactive to the GP33 epitope of LCMV. Note that, although aged P14 mice had a clear population of CD44hi DGP33 tetramer-positive CD8 T cells, all PD1 expressing CD8 T cells in these mice were tetramer negative (Supplemental Fig. 2). This observation suggests the use of a nontransgenic TCR and perhaps a role for TCR stimulation in age-related increases in PD1 expression. We sorted these P14 CD8 T cells into two groups on the basis of CD44: CD44lo (naïve D8 T cells) and CD44hi (memory-like CD8 T cells, Fig. 6A). To test the impact of a shift from CD44lo to CD44hi CD8 T cells with age, equal numbers of either young or aged CD44lo and CD44hi P14 cells were purified and adoptively transferred to young congenic recipient mice that were subsequently challenged with LCMV (Fig. 6C). Although there was clearly a hierarchy in the responsiveness of these different donor populations (Fig. 6D, 6E), the CD44hi P14 cells from aged mice were 10- to 20-fold less efficient in the initial expansion and accumulated poorly in the blood compared with the other populations. Also, a lower percentage of these CD44hi aged donor cells incorporated BrdU compared with young P14 cells (17.3 ± 1.2 versus 26.7 ± 1.9%; p = 0.0056; Fig. 6F, 6G). Despite perhaps a slight disadvantage of CD44lo CD8 T cells from aged mice in expansion (Fig. 6F), we found similar BrdU incorporation between young CD44lo and aged CD44lo cells (∼29.6 ± 2.3 and 34.4 ± 2.3%, respectively). We also observed that, in the spleen, CD44hi P14 cells from young donors had moderately diminished responses compared with CD44lo cells from same donors though...
The percentages of CD44 Hi CD8 T cells that produce IFN-γ, IL-2, and MIP1α staining are shown in A. Graphs in B show absolute numbers of young and aged naive CD8 T cells that produce IFN-γ, IL-2, and MIP1α. Representative plots of CD8 T cells show IFN-γ production versus CD44 expression (C). The percentages of CD44Hi CD8 T cells that produce IFN-γ are shown in D. E is gated on CD8 T cells from young and aged mice and shows IFN-γ versus PD1 expression. Data are representative of two independent experiments including eight to nine mice per experiment. *0.05 > p > 0.01, **0.01 > p > 0.001, ***p < 0.001 by unpaired two-tailed t test.

CD44Hi P14 cells from aged mice clearly had the most defective proliferative expansion of the populations examined (Fig. 6F, 6G). Previous studies indicated that CD44Lo CD8 T cells generated by homeostatic proliferation in lymphopenic hosts responded more vigorously in response to bacterial challenge compared with CD44Lo CD8 T cells (10). We therefore extended our studies of naturally arising CD44Hi CD8 T cells from young mice to another setting. KOVA-specific CD8 T cells were isolated from uninfected young OT1 TCR transgenic mice on Rag2−/− background (OT1 Rag2−/− mice). In OT1 Rag2−/− mice, all CD8 T cells are OVA specific. We sorted these CD8 T cells into CD44Lo (naive CD8 T cells) and CD44Hi (memory-like CD8 T cells; Fig. 6F). Equal numbers of purified CD44Lo and CD44Hi OVA-specific CD8 T cells from young mice were adoptively transferred to young congenic recipient mice, and those recipients were infected with vesicular stomatitis virus expressing OVA. Once again, naturally arising CD44Hi cells from young mice were 3- to 4-fold less efficient in the initial expansion and accumulated to lower frequency and absolute number in the blood and spleen compared with the CD44Lo populations (Fig. 6J, 6K). Thus, using two different TCR transgenic models and two different types of infections, we demonstrated that although CD44Hi CD8 T cells from young mice can serve as precursors, their response to infection is reduced compared with responses that arise from CD44Lo precursors. These data suggest that at least part of the reason for poor antiviral CD8 T cell responses in old mice is due to the non–lymphopenia-induced natural shift of some Ag-specific precursors to a CD44Hi phenotype.

Discussion

Aging-related susceptibility to infectious diseases and cancer is associated with diminished immune function including reduced T cell responses. Although some aspects of declining T cell responses with age reflect systemic changes (e.g., an increase in regulatory T cells, etc.), others are due to cell-intrinsic defects (45). In the current study, we demonstrated both quantitative and qualitative age-related changes in T cells and found that these changes were linked to defective responses upon viral infection. The decrease in peripheral T cell numbers likely reflects thymic involution that starts early (perhaps as early as 6 wk in mice and age 1 in humans) and continues through life (3, 46). Although some functional thymic tissue can be found at old age (47), T cell numbers in the periphery are thought to be retained increasingly by homeostatic proliferation as thymic output declines. Both ho-
meostatic proliferation and exposure to environmental Ags are believed to be responsible for the phenotypic changes in T cells that occur with age. Indeed, T cells that undergo a homeostasis driven-proliferation under lymphopenic conditions have been shown to upregulate CD44 (8, 48). Interestingly, CD44Hi T cells can be also found in germ-free mice (9, 49), suggesting that these cells can arise during physiological lymphopenia in the process of aging in the absence of commensal microbes.

CD44Hi CD8 T cells from uninfected aged mice express high levels of several inhibitory receptors including PD1, LAG3, 2B4, and CD160. Upregulated expression of inhibitory receptors, such as PD1, is typically found on T cells responding to persisting Ag stimulation and chronic infection in both mice and humans, but PD1 and related pathways also have a central role in regulating peripheral self-tolerance and autoimmunity (50, 51). Recent studies showed that PD1 expression correlates with impaired function of LCMV-, HIV-, or HCV-specific CD8 T cells and that the blockade of PD1 and/or other inhibitory receptors results in improved CD8 T cell functionality (39). Furthermore, CD4 T cells from aged mice can also express increased PD1 (19), and PD1-positive CD4 T cells from aged mice exhibit proliferative hypo-

responsiveness in vitro (20) and in vivo (52). Blockade of PD1/PDL1 pathway in vitro moderately improved cytokine production, but did not restore proliferation of PD1-positive T cells from aged mice (21). The impact of high expression of PD1 on CD8 T cells during aging remains less well defined. In addition, PD1 is clearly not the only inhibitory receptor expressed by these cells, and as occurs during chronic infections, coexpression of inhibitory receptors might influence the function of these aged cells. In combination with microarray studies, these observations suggest a global age-associated change in gene expression in CD8 T cells from uninfected mice that has similarities to what occurs for exhausted T cells from aged mice.

**FIGURE 6.** Poor responsiveness of Ag-specific CD44Hi CD8 T cells from uninfected aged and young mice. P14 (D^dp33 specific) CD8 T cells were purified from uninfected young and aged P14 mice (Ly5.1^+) and sorted, based on the expression of CD44 (A). B, D^dp33 tetramer staining on sorted P14 populations. After sorting, equal numbers of young and aged CD44Hi and CD44Lo P14 cells were transferred into recipient mice (Ly5.2^+). Recipient mice were then challenged i.p. with LCMV (2 \* 10^6 PFU/mouse, C). The responses of the donor P14 CD8 T cells were monitored in the blood (D) and spleens (day 8 postinfection, E-G) of recipient mice. Lower frequencies of CD44Hi P14 CD8 T cells derived from aged and young donors were observed compared with their CD44Lo counterparts. E and F, The absolute number and frequency of BrdU^+ donor P14 CD8 T cells derived from young and aged CD44Hi and CD44Lo cells in the spleens of recipient mice at day 8 postinfection. In these experiments, 12 h preanalysis, mice were injected with BrdU i.p. Data are representative of two independent experiments including four to five mice per experiment. Similarly, OT1 (K^bOVA specific) CD8 T cells were purified from uninfected young OT1 Rag2^−/− mice (Ly5.1^+) and sorted, based on the expression of CD44 (H). I, K^bOVA tetramer staining on sorted OT1 populations. After sorting, equal numbers of young CD44Hi and CD44Lo OT1 cells were transferred into recipient mice (Ly5.2^+). Recipient mice were then challenged i.v. with VSV-OVA (2 \* 10^6 PFU/mouse). The responses of the donor OT1 CD8 T cells were monitored in the blood (day 7 postinfection, J) and spleens (day 8 postinfection, K) of recipient mice. Lower numbers of CD44Hi OT1 CD8 T cells (○) were observed compared with CD44Lo OT1 CD8 T (○). *p < 0.05, **p < 0.01, ***p < 0.001 by unpaired two-tailed t test.
CD8 T cells. In addition to inhibitory receptors, these gene expression profiles also identified a number of other immunoregulatory and/or transcriptional pathways that differed between CD8 T cells from young and aged mice. These differences included components of AP-1 (e.g., *Fos*, *Fosb*, *Jun*, and *JunB*), other transcription factors (*Myb*, *Klf4*, *Maf*, and *Prdm1*), and *IL10* and *Fasl*. It will be interesting to investigate the importance of these transcriptional changes in the altered responses of T cells during aging.

An interesting question that arises is, what is the cause for accumulating inhibitory receptor expression with age. Despite the similarities to T cells found during chronic infections, it is important to note that our aged mice were consistently negative for all common rodent pathogens including viruses such as LCMV and MNV. This information suggests that, although signatures of T cell exhaustion might reflect exposure to unknown infectious agents, other age-related events (such as increased self-reactivity) might underlie the inhibitory receptor expression and other transcriptional changes. Indeed, T cells undergoing recognition of self-Ag in vivo express PD1 during the induction of tolerance (38). Although most of these tolerized T cells are typically physically deleted in young mice, we propose that during aging some of these tolerized T cells escape deletion, persist, and accumulate over time. Oligoclonally expanded T cells have also been previously shown to express PD1 (40). Although we observed some oligoclonal expansion of the CD8 T cells in uninfected aged mice, these expansions could not explain all of the PD1 expression, because the percentage of oligoclonally expanded cells was typically <10–15%, whereas 20–60% of CD8 T cells expressed PD1. It is interesting that PD1-positive cells were found predominantly in the polyclonal pool of CD44HI CD8 T cell from naive mice. Although tetramer-positive CD44HI cells from aged P14 transgenic mice were PD1 negative, these mice did contain a population of tetramer-negative CD8 T cells that were PD1 positive (Supplemental Fig. 2). It is possible that tetramer-negative CD8 PD1HI T cells in these P14 mice cross-react with self- or environmental Ags. Future studies are necessary to test this idea, but if true, it could suggest that the pool of Ag-specific CD8 T cells in aged mice prior to infection (i.e., CD44HI PD1+ CD8 T cells) would be more cross-reactive compared with the CD8 T cell pool in young animals.

It is interesting to note, however, that even for the CD44HI PD1-lo CD8 T cells from naive aged animals, responses were qualitatively inferior compared with CD44Lo CD8 T cells. We also observed diminished proliferative responses of naturally arising CD44HI cells from young transgenic animals compared with CD44Lo cells. These observations were somewhat surprising given the work by others demonstrating enhanced responsiveness of homeostatically generated CD44HI CD8 T cells (9, 10). Taken together, these data suggest that naturally arising CD44HI CD8 T cells that accumulate with age might be functionally distinct from those generated by strong homeostatic emptiness following adoptive transfer of CD44Lo T cells into lymphopenic hosts (e.g., over ~3–4 wk in irradiated or Rag−/− mice). One possible explanation for some of these differences could be that transient bacterial release after sublethal irradiation influences the functional quality of CD44HI memory cells; if these CD44HI cells are generated in antibiotic-treated hosts, they show dramatic impairment in protective immunity against bacterial infections (53). In addition, the decreased ability of CD44HI precursors to respond to infection is consistent with the changes in global gene expression in these cells, including the prominent increase in multiple cell intrinsic (e.g., PD1) and potentially cell extrinsic (e.g., *IL-10*) negative regulatory pathways. The global shift to CD44HI CD8 T cells with age is obviously associated with the accumulation of functional, Ag-induced memory T cells generated by infection or vaccination. However, our data now suggest that the shift of some naive precursors into the CD44HI pool in the absence of overt Ag stimulation or severe lymphopenia is associated with reduced proliferative capacity and potentially other functional defects. These observations suggest the possibility that generating high-quality memory T cells prior to this shift to defective precursors (i.e., when young) might enhance immunity into old age. Studies to directly test this notion are under way.

Another key finding of the current study was that the precursor frequency of Ag-specific CD8 T cells present prior to infection was 10-fold lower in aged compared with young mice. Previous studies demonstrated that age-associated changes in immunodominance could be due to TCR repertoire changes and/or low precursor frequency leading to holes in the repertoire (15). We have quantified this loss of virus-specific precursors for two different Ag specificities and further demonstrated that many Ag-specific precursors from uninfected aged mice are CD44HI and express PD1. The decrease in both the number and quality of Ag-specific precursors in aged mice could mean that these cells must undergo greater numbers of divisions upon infection to achieve the same clonal expansion causing a delay in the early antiviral effector CD8 T cell response in aged mice consistent with previous observations (24). This delay in clonal expansion during an acute viral infection could result in a substantial increase in viral replication and dissemination. Moreover, the increased number of divisions could also result in defects in effector and memory T cell responses, drive terminal differentiation or even skew the responding T cells toward functional exhaustion. The expression of PD1 prior to infection could mean even further difficulties in proliferation, expansion and for functionality of these cells and enhanced PD1 signaling could alter CD8 T cell differentiation through induction of transcriptional pathways such as basic leucine zipper transcription factor, ATP-like (54).

Thus, we demonstrate in this study that the ability of CD8 T cells from young and aged mice to respond to infection is at least partially influenced by intrinsic and population-based changes in CD8 T cells. Precursor frequency plays a significant role in T cell memory quality (55), and our data now demonstrate major changes in Ag-specific CD8 T cell precursor frequency are associated with age-dependent defects in antiviral immunity. Understanding these aging-associated changes could help define the molecular mechanisms underlying defective CD8 T cell responses. Strategies that result in vigorous production of new CD44Lo truly naive T cells from the thymus, prevention of T cell senescence, and modulation/manipulation of immunoregulatory pathways are some of the attractive approaches that could enhance immunity and responses to vaccines in the elderly.

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**Disclosures**

The authors have no financial conflicts of interest.

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