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Although previous studies have demonstrated delayed viral clearance and blunted effector T cell responses in aged mice during infection, memory CD8 T cells and especially secondary responses have received less attention. In this study, we show that modest differences in the number of memory CD8 T cells formed in aged versus young animals were associated with altered memory CD8 T cell differentiation. Aged immune mice had increased morbidity and mortality upon secondary viral challenge, suggesting changes in T cell immunity. Indeed, virus-specific memory CD8 T cells from aged mice showed substantially reduced proliferative expansion upon secondary infection using multiple challenge models. In addition, this defect in recall capacity of aged memory CD8 T cells was cell-intrinsic and persisted upon adoptive transfer into young mice. Thus, the poor proliferative potential of memory T cells and altered memory CD8 T cell differentiation could underlie age-related defects in antiviral immunity. ***The Journal of Immunology*****, 2010, 184: 5151–5159.**

Loss of effective immune responses with age has a substantial impact on health and survival (1). As a consequence of diminished immune function, humans older than 65 y are more susceptible to infections (e.g., influenza, tuberculosis) (2, 3), have higher reactivation rates of persistent viruses (e.g., varicella-zoster virus) (4), and respond less effectively to vaccination (5–9). The inability of elderly populations to rapidly control or eliminate infections not only increases their risk for serious sequalae of the primary infections, but also can contribute to a decline in health on multiple levels caused by events including increased risk of secondary infections and/or cardiovascular complications (10).

Alterations in both innate and adaptive immunity have been observed with age (11). Changes in neutrophils, macrophages, NK cells (11), pathogen recognition via TLRs (12), and the subsequent production of cytokines by cells of the innate immune system (13, 14) have all been reported in aged mice or elderly humans and could influence immunity to infection. Changes also occur in both B and T cell responses during aging (15). These changes include a global shift from naive to memory-phenotype T cells resulting in a decrease in the number of naive T cells and an increase in T cells of memory phenotype (16–19), clonal expansions of memory-phenotype T cells in mice, primates, and humans (18, 20–22), and changes in regulatory T cell (Treg) numbers (23, 24). Although new thymic emigrants in aged mice appear more functional than their counterparts that have aged in the periphery (25), the dramatic decrease in thymic output with age leads to a reduced peripheral naive CD4 and CD8 T cell pool with age. Ag-specific T cells themselves can also be intrinsically altered with age. For example, CD4 T cells from aged animals proliferate or produce cytokines poorly (e.g., IL-2, IL-10) compared with CD4 T cells from young animals (15, 26, 27) and have altered TCR signaling (28, 29). Aged CD4 T cells also provide decreased help for humoral responses (30).

Compared to CD4 T cells, the effects of aging on Ag-specific CD8 T cells have not been as extensively examined, and available data are conflicting. Although some studies reported that CD8 T cell responses are negatively affected in older animals both in vitro (31, 32) and in vivo (33–35) or that T cell repertoire diversity and effector functions are altered (19, 36, 37), other data suggest that aged CD8 T cells are not intrinsically defective (38, 39). In response to viral infection, CD8 T cell responses have been assessed quantitatively (33, 36), and, although numerical decreases have been reported (37), it remains unclear if there are also qualitative differences between antiviral CD8 T cells from young or aged mice. In addition, most studies that have examined antiviral immunity with age have examined primary infection, and there is little information about the quantitative or qualitative aspects of T cell memory or T cell responses upon re-encounter with pathogens in an aged immune host. If deficiencies in antiviral memory CD8 T cell responses do exist with age, it will be critical to define the nature of these defects to begin to understand how they contribute to changes in response to reinfection and to determine the underlying pathways.

We have investigated these issues by examining the properties of memory cells formed in aged and young mice following infection with either lymphocytic choriomeningitis virus (LCMV) or influenza virus. The number of memory CD8 T cells generated in the aged and young mice was similar or decreased in the old mice (depending of the epitope tested), but the pattern of memory T cell differentiation was altered. In addition, LCMV and influenza virus-specific memory CD8 T cells in aged mice were highly compromised in their ability to undergo proliferative expansion upon secondary infection. The low proliferative capacity of memory

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Abbreviations used in this paper: A, aged; Arm, Armstrong; i.n., intranasal(ly); LCMV, lymphocytic choriomeningitis virus; p.i., postinfection; Treg, regulatory T cell; VV, vaccinia virus; VVNP396, vaccinia virus expressing lymphocytic choriomeningitis virus NP396 epitope; Y, young.

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CD8 T cells from aged mice upon viral challenge was at least partially cell-intrinsic because these defects were observed even following adoptive transfer into young mice. Thus, our studies demonstrated that cell-intrinsic changes in proliferative capacity contribute to suboptimal memory CD8 T cell responses in the aged mice and hence may help explain the increased susceptibility to viral infections in the elderly.

Materials and Methods

Mice and viruses

Young C57BL/6 and Ly5.1 mice (2–4 mo old) were purchased from the National Cancer Institute (Frederick, MD) or Taconic (Germantown, NY). Aged C57BL/6 mice (18–22 mo old) were obtained from the National Institute on Aging (Bethesda, MD) or The Wistar Institute aging colony (Philadelphia, PA) (purchased from Taconic when young and then aged). For all adoptive transfer experiments, congenic mice differing in Ly5 (Ly5.1 versus Ly5.2) were used. All mice were used in accordance with Institutional Animal Care and Use Committee guidelines. Aged animals exhibiting enlarged spleens and tumors were excluded from the studies.

For primary infections, mice were inoculated with either LCMV Armstrong (LCMV Arm; 2×10^5 PFU) i.p. or recombinant influenza virus expressing the LCMV GP33 epitope (X31-GP33; 1.6×10^5 50% tissue culture infective dose) intranasally (i.n.). For rechallenge infection, mice were infected with: 1) PR8-GP33 (3 LD_{50} i.n.); 2) vaccinia virus (VV) expressing LCMV NP396 epitope (VVNP396; 3×10^5 PFU i.n.); 3) VV expressing influenza virus NP366 epitope (VVNP366; 3×10^5 PFU i.n.); or 4) X31-GP33 (1×10^5 50% tissue culture infective dose i.n.). Prior to i.n. infections, mice were anesthetized by i.m. injection of ketamine hydrochloride and xylazine (Phoenix Scientific, San Marcos, CA) in 0.2 ml Life Technologies HBSS (Invitrogen, Carlsbad, CA). Recombinant influenza strains containing the LCMV GP33–41 epitope inserted in the neuraminidase stalk region were obtained from Dr. Richard J. Webby (St. Jude Children's Research Hospital, Memphis, TN), propagated in eggs, and stored at −80°C. The replication and pathogenicity of these recombinant strains of X31 and PR8 were not substantially different from their nonrecombinant counterparts (data not shown). Viral titers were determined by plaque assay on Vero cell monolayers (for LCMV and VV) or on Madin-Darby canine kidney cell monolayers (for X31-GP33 and PR8-GP33) as previously described (40, 41).

Adoptive transfer

CD8 T cells were purified (>90% purity) using magnetic beads ($CD8^+$ T cell isolation kit, MACS beads; Miltenyi Biotec, Auburn, CA). Between 7 and 56×10^3 DbNP396- or DbNP366-specific CD8 T cells or 25–50 $\times$ 10^3 DbGP33-specific CD8 T cells were transferred i.v. (in each individual experiment, identical numbers of DbNP396, DbNP366, or DbGP33 $tetramer^+$ CD8 T cells from young versus aged donors were adoptively transferred to each recipient mouse). Donor populations were monitored in the peripheral blood by retro-orbital blood collection as described previously (40).

Isolation of lymphocytes from tissues

Lymphocytes were isolated from tissues as described (40). Briefly, mice were euthanized and the hepatic vein cut. Liver and lungs were perfused by injection of PBS into hepatic artery or the right heart ventricle, respectively. Lungs were cut into pieces and incubated in 0.2 mg/ml collagenase D (Roche Diagnostic, Indianapolis, IN) at 37°C for 35 min. Livers were homogenized and incubated in 0.25 mg/ml collagenase D (Roche Diagnostic) and 1 U/ml DNase I (Roche Diagnostic) at 37°C for 45 min. Digested livers were applied to a 44%/56% Percoll gradient and centrifuged at $850 \times g$ for 20 min at room temperature, and the lymphocyte population was harvested from the interface. Spleens and lymph nodes were homogenized using a cell strainer. In all tissues, RBCs were lysed using ACK lysing buffer (Quality Biologicals, Gaithersburg, MD), and lymphocytes were washed and counted.

Histology/pathology

In some experiments, samples from the same lungs were used to analyze virus titers and histology. In these experiments, the left lobe was used for histology, whereas the remaining lobes were used to test for virus titers.

The left lobe was gently inflated with 100 μl of a 10% formalin solution through a 23-gauge needle. The inflated lung samples were then directly submerged in 10% formalin solutions for tissue fixation at 4°C for 24 h. Formalin-fixed lung samples were paraffin-embedded and sectioned at 4 μm for mounting on microscope slides. Section slides were subsequently stained with Harris hematoxylin (Thermo Fisher Scientific, Waltham, MA) and alcoholic eosin according to the manufacturer's recommended protocol.

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 (40). Abs to CD8, CD44, IFN-γ, TNF, and programmed death-1 (RPMI-30) were purchased from eBioscience (San Diego, CA). The Ab to granzyme B was purchased from Caltag Laboratories (Burlingame, CA). All other Abs were purchased from BD Biosciences (San Jose, CA). Staining and analysis were performed as previously described (40).

Function was investigated by intracellular cytokine staining following Ag stimulation (IFN-γ, IL-2, CD40L, TNF). Briefly, 1×10^6 splenocytes were cultured in the absence or presence of the indicated peptide (0.2 μg/ml) and brefeldin A for 5 h at 37°C. Following staining for surface Ags as described above, cells were stained for intracellular cytokines 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 they were sacrificed. BrdU staining was carried out using a BrdU Flow Kit (BD Pharmingen, San Diego, CA) in accordance with the manufacturer's instructions. BrdU was detected using FITC-conjugated anti-BrdU Ab.

Samples were collected using an FACSCalibur or LSR II flow cytometer (BD Biosciences).

Statistical analysis

Data were analyzed using a two-tailed Student t test, and a p value of ≤ 0.05 was considered significant.

Results

Antiviral memory CD8 T cell responses were compared in young (~2–4 mo) versus aged (~18–20 mo) mice using two different models of viral infection: systemic infection with noncytopathic LCMV Arm and localized i.n. infection with cytopathic influenza virus. Infection with LCMV is a well-studied system in which the virus itself does not normally cause pathology, and CD8 T cells are crucial to control infection (42). Influenza virus is known to cause a severe infection in elderly humans, and influenza vaccines are less effective in older adults (9). Influenza virus is cytopathic, and although many arms of the immune response can contribute to control of primary influenza virus infection (e.g., CD8 T cells, CD4 T cells, B cells, NK cells), CD8 T cells have been a considerable focus of attention for heterotypic protection from reinfection. In the studies described below comparing the responses in young and aged mice to these two viruses, the use of a recombinant strain of influenza virus expressing the GP33 epitope of LCMV (X31-GP33) allowed the comparison of the responses to the same viral epitope following either systemic or local infection in some experiments.

Virus-specific memory CD8 T cell populations in aged versus young mice

The number of Ag-specific memory CD8 T cells was determined in different tissues of LCMV and influenza virus immune mice at 1 to 2 mo postinfection (p.i.) using MHC class I tetramer staining (Fig. 1). Following LCMV infection of aged mice, a modestly blunted effector CD8 T cell response was observed consistent with previous reports (Fig. 1*A*, 1*B*, and data not shown) (33). However, memory CD8 T cells were generated and maintained in the blood and tissues for at least 45 d (Fig. 1*A–D*). Although the frequency of the memory DbGP33 $tetramer^+$ CD8 T cells was slightly elevated in the blood of aged mice compared with young mice (aged mice: $1.49 \times 10^4 \pm 3.83 \times 10^3$ cells/10^6 PBMC; young mice: $4.55 \times 10^3 \pm 8.09 \times 102$ cells/10^6 PBMC), the total number of DbGP33 $tetramer^+$ CD8 T cells in the spleen was similar between these mice (aged mice: $2.01 \times 10^5 \pm 4.53 \times 10^4$ cells/spleen; young mice: $2.05 \times 10^5 \pm 4.13 \times 10^4$ cells/spleen; Fig. 1*A*, 1*D*).

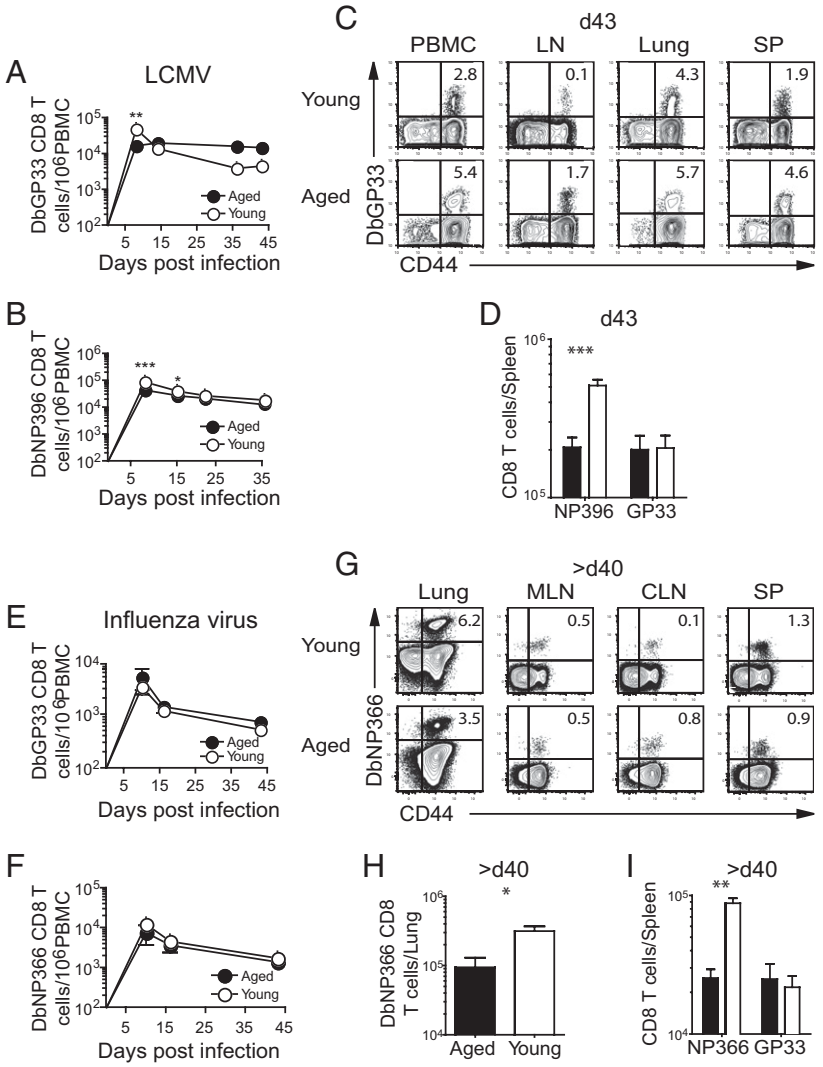

FIGURE 1. Advanced age moderately affects the overall magnitude of antiviral memory CD8 T cell populations. Ag-specific CD8 T cell responses following LCMV Arm (*A*, *B*) or influenza virus (*E*, *F*) infection of aged (black symbols) and young (white symbols) mice were monitored longitudinally in the PBMCs of individual mice. The frequency of Ag-specific memory CD8 T cells in different tissues of LCMV Arm- (*C*) and influenza virus-infected (*G*) aged and young mice was determined by tetramer staining. Plots are gated on CD8 T cells, and numbers indicate the percentages of tetramer$^+$ CD8 T cells. The numbers of Ag-specific CD8 T cells in the spleens of LCMV Arm-immune (*D*, d43 p.i.) and in the lungs and spleens of influenza virus-immune (X31-GP33; *H*, *I*, >d40 p.i.) aged (black bars) and young (white bars) mice determined by tetramer staining are shown. Error bars (*D*, *H*, *I*) represent SEM. Data are representative of three to five independent experiments including 8–10 mice/experiment. *$0.05 > p > 0.01$; **$0.01 > p > 0.001$; ***$p < 0.001$ by unpaired two-tailed *t* test.

In contrast, the frequency of the memory DbNP396 tetramer$^+$ CD8 T cells in the blood of young mice and aged mice was similar (aged mice: $1.25 \times 10^4 \pm 2.97 \times 10^3$ cells/10^6 PBMC; young mice: $1.87 \times 10^4 \pm 2.84 \times 10^3$ cells/10^6 PBMC), but the absolute number of CD8 T cells specific for this immunodominant DbNP396 epitope was reduced in the spleens of aged animals (aged mice: $2.08 \times 10^5 \pm 3.2 \times 10^4$ cells/spleen; young mice: $5.1 \times 10^5 \pm 4.58 \times 10^4$ cells/spleen; Fig. 1*B*, 1*D*). At the memory time point, the aged mice had on average $6.3 \times 10^6 \pm 1.97 \times 10^6$ CD8 T cells/spleen, whereas spleens of young LCMV-infected mice contained $10.3 \times 10^6 \pm 5.76 \times 10^5$ CD8 T cells (data not shown). Although blood collection represents a valuable tool to follow the progression of infection, only the frequency of Ag-specific cells can be determined. On the other hand, data from tissues reflect both the frequency and the number of Ag-specific CD8 T cells. The discrepancy between blood and spleen could reflect a change in recirculation of Ag-specific cells in old mice, but also could simply be due to differences in frequency versus absolute numbers.

Influenza virus-specific memory CD8 T cell responses were generated at similar frequencies in the blood of young and aged mice (Fig. 1*E*, 1*F*). At memory time points, the total numbers of DbGP33 and DbPA224 tetramer$^+$ CD8 T cells were similar in aged versus young mice (Fig. 1*I* and data not shown). However, although the mediastinal and cervical lymph nodes contained similar numbers of CD8 T cells specific for the immunodominant DbNP366 epitope in young and aged mice (data not shown), the absolute number of DbNP366-specific CD8 T cells was significantly reduced in the lungs and spleens of aged animals (Fig. 1*G–I*). The spleen and lung represent the primary sites of viral replication for LCMV and influenza virus, respectively. Thus, it is possible that the greatest impact on absolute number of Ag-specific CD8 T cells will therefore be observed in these locations. Alternatively, differences in homing and migration might exist. It should be also noted that although plots in Fig. 1*G* (gated on CD8 T cells) show different frequencies of DbNP366-specific CD8 T cells in cervical lymph nodes, the difference in total cellularity of these lymph nodes between aged and young mice accounts for similar total numbers of DbNP366-specific cells in aged and young mice.

Memory CD8 T cell populations were generated following LCMV or influenza virus infection in aged mice with moderate influence on the overall magnitude, particularly a significant reduction in immunodominant CD8 T cell responses, whereas subdominant responses appeared less impaired by age. The observed difference in the responses to immunodominant and subdominant epitopes could reflect the differences in precursor frequency between aged and young mice as recently reported by Yager et al. (19).

Memory CD8 T cell quality in aged versus young mice

To investigate whether the memory CD8 T cell populations in aged mice differed qualitatively from those generated in young mice, we examined tetramer$^+$ CD8 T cells using a panel of phenotypic

markers associated with memory CD8 T cell differentiation (Fig. 2). The expression of molecules such as CD62L, CD127, and CD27 is associated with development of robust and highly responsive memory T cells (43–45), and coexpression of these molecules can identify subpopulations of developing memory T cells with different properties (43, 45–47). Although there was a little difference in the expression of granzyme B or KLRG1, fewer LCMV-specific memory CD8 T cells in aged mice were CD62L^{hi}, CD127^{hi}, or CD27^{hi} cells compared with LCMV-specific CD8 T cells in young mice (Fig. 2*A*, 2*B*). Indeed, CD62L and CD27 coexpression patterns were markedly different for LCMV-specific memory CD8 T cells from aged versus young mice (Fig. 2*B*). Also, fewer DbGP33-specific memory CD8 T cells generated following LCMV or influenza virus infection in aged mice were CD62L^{hi}, indicating an impact on memory CD8 T cells differentiation in aged mice during two different viral infections (Fig. 2*C*). These differences might be consistent with a delay in the generation of central memory-like CD8 T cells in the aged mice and a shift toward more effector memory-like and/or senescent T cells.

To examine the functional competence of memory CD8 T cells in aged mice, we compared the number of tetramer$^+$ memory cells to the number of Ag-specific CD8 T cells that were capable of producing IFN-γ in response to the same peptide. Although ~100% of tetramer$^+$ CD8 T cells from young LCMV-immune mice could produce IFN-γ in 5 h, nearly half of the DbNP396-specific CD8 T cell population in aged mice failed to produce this cytokine efficiently following Ag stimulation (Fig. 3*A*, 3*B*). In addition, the ability of LCMV-specific memory CD8 T cells from aged mice to produce IL-2 or upregulate CD40L was compromised compared with young mice (Fig. 3*C*–*E*). This defect in dual production of IFN-γ/IL-2 or IFN-γ/CD40L was true for both DbNP396- and DbGP33-specific CD8 T cells. In contrast to LCMV-specific memory CD8 T cells, influenza virus-specific CD8 T cells in young and aged mice were equally functional (Fig. 3*F*). In the spleens of influenza virus immune mice, all tetramer$^+$ CD8 T cells (DbNP366, DbPA224, and DbGP33) were capable of producing IFN-γ in 5 h (Fig. 3*F* and data not shown). In addition, similar percentages of IFN-γ–producing cells were able to coproduce TNF and IL-2 and express CD40L between aged and young mice (Fig. 3*F* and data not shown). Thus, although there are some modest changes in memory CD8 T cell populations, especially for those responding to dominant epitopes, overall memory CD8 T cells from aged mice are generated and are either functionally competent for cytokine production or have modest defects in eliciting rapid effector functions. The precise functional characteristics of memory CD8 T cell populations in aged mice might depend on the immunodominance of the epitope or the type of infection.

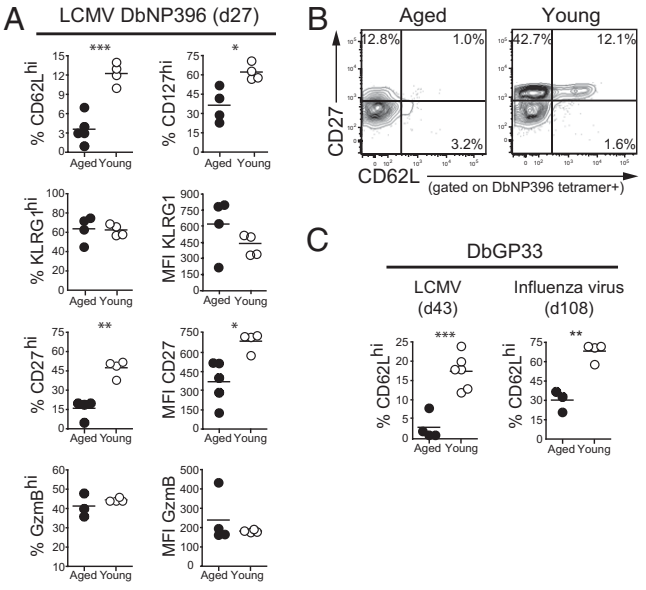

FIGURE 2. Altered expression of phenotypic markers of normal memory T cell differentiation in aged mice following viral infection. The phenotype of Ag-specific memory CD8 T cells was compared between aged (black symbols) and young (white symbols) mice during LCMV (*A*–*C*, days 27 and 43 p.i.) and influenza virus (*C*, day 108 p.i.) infections. Ag-specific memory CD8 T cells were identified by tetramer staining and costained using a panel of phenotypic markers associated with memory CD8 T cell differentiation. Plots in *B* are gated on DbNP396-specific memory CD8 T cells (day 30+ post LCMV infection), and numbers indicate the percentages of different subpopulations. Data are representative of three independent experiments including 8–10 mice/experiment. *$0.05 > p > 0.01$; **$0.01 > p > 0.001$; ***$p < 0.001$ by unpaired two-tailed *t* test.

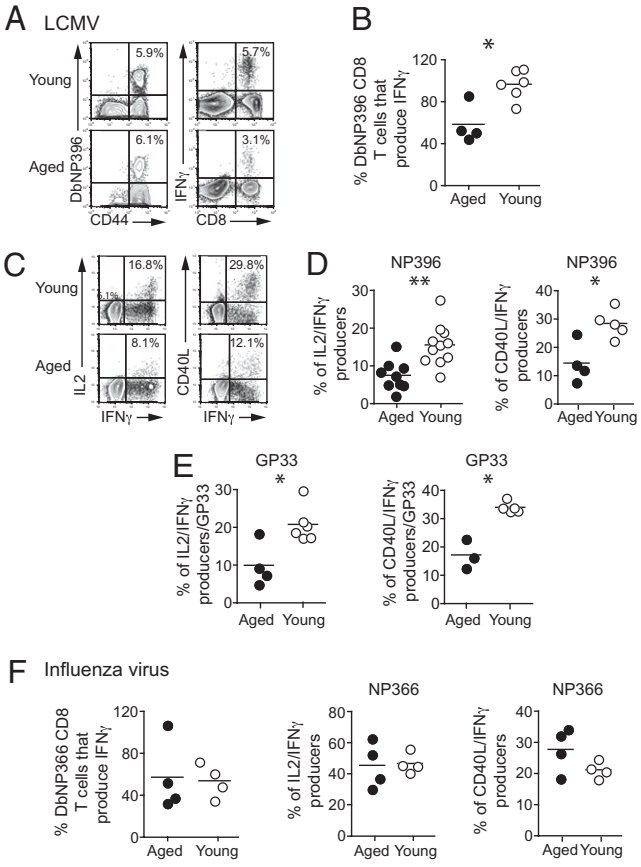

FIGURE 3. Memory CD8 T cells from aged mice have changes in functionality compared with memory CD8 T cells from young mice following LCMV but not influenza virus infection. The percent of LCMV DbNP396 tetramer$^+$ CD8 T cells that were capable of producing IFN-γ was determined by comparing tetramer staining to intracellular cytokine staining on day 43 p.i. (*A*, *B*). Function was further investigated by examining coproduction of IFN-γ and IL-2 or CD40L by intracellular cytokine staining following Ag stimulation. LCMV DbNP396-specific (*C*, *D*) and GP33-specific (*E*) CD8 T cells from aged mice had decreased IFN-γ and IL-2 or IFN-γ and CD40L coexpression compared with young mice. Similar analyses performed on CD8 T cells from spleens on day 50 after influenza virus (X31-GP33) infection revealed little difference in the ability to produce or coproduce cytokines in aged and young mice (*F*). *$0.05 > p > 0.01$; **$0.01 > p > 0.001$ by unpaired two-tailed *t* test.

Protective immunity in aged and young mice

To investigate the capacity of memory CD8 T cells generated in aged animals to respond to secondary infections, we designed a series of rechallenge experiments. First, young and aged X31-GP33 immune mice were challenged with heterologous PR8-GP33 virus (Fig. 4*A*). Protection in this model is thought to depend on T cell immunity because X31 and PR8 have different hemagglutinin and neuraminidase proteins. Although young mice were fully protected, PR8-GP33 infection of the aged X31-GP33 immune mice resulted in substantial morbidity and mortality (Fig. 4*B*, 4*C*). Approximately 30% of aged mice succumbed to PR8-GP33 infection, whereas all young mice survived this dose of virus (Fig. 4*C*). Also, at early time points post rechallenge infection, higher viral titers were observed in the lungs of aged compared with young mice (Fig. 4*D* and data not shown). The majority of aged mice still harbored high levels of replicating virus in the lungs at day 5 post rechallenge. At the same time, virus was completely cleared from the lungs of young mice. In addition, the number of circulating DbNP366 tetramer$^+$ CD8 T cells on day 6 post rechallenge was significantly decreased in aged compared with young mice (Fig. 4*E*). The numbers of DbGP33 tetramer$^+$ cells were similar between aged and young mice (Fig. 4*F*), but this DbGP33-specific CD8 T cell response also peaked later than the dominant DbNP366-specific CD8 T cell response. Furthermore, marked differences were observed in lung pathology between aged and young animals. The lungs of aged mice showed substantially higher inflammatory infiltrate consistent with greater morbidity and mortality in these mice (Fig. 4*G*). Although many components of the immune responses and even immunopathology might underlie these distinct outcomes of secondary infection in young and aged immune mice, these observations also suggest a potential difference in T cell immunity.

Defects in the secondary response of memory CD8 T cells from aged mice are cell-intrinsic

Examining the quality of memory CD8 T cells using direct rechallenge of immune hosts is complicated by several variables that might influence the response (e.g., host age, Ab, innate immune responses, antigenic load, etc.). To distinguish the potential effects of the aged host from the intrinsic quality of memory CD8 T cells from aged mice, memory CD8 T cells from young or aged LCMV- or influenza virus-immune mice were isolated and adoptively transferred to young congenic recipient mice followed by rechallenge (Fig. 5*A*). The number of LCMV DbNP396-specific or influenza virus DbNP366-specific memory CD8 T cells from young or aged mice (Ly5.2$^+$) was normalized prior to adoptive transfer to allow direct per-cell comparison. One day posttransfer, recipient mice were challenged i.n. with VV expressing the LCMV DbNP396 or the influenza virus DbNP366 epitope for recipients of LCMV-specific memory versus influenza virus-specific memory CD8 T cells, respectively (Fig. 5*A*). Donor DbNP396 (Fig. 5*B*, 5*D*) or DbNP366-specific (Fig. 5*C*, 5*E*) CD8 T cell responses were monitored in the blood over time. Memory CD8 T cells specific for LCMV or influenza virus from aged mice were both severely compromised in their ability to mount a robust recall response compared with memory CD8 T cells from young mice (Fig. 5*B*–*G*). In the blood, ~3–10-fold lower frequencies of virus-specific CD8 T cells derived from aged donors were observed at most time points examined compared with their young counterparts. When sacrificed ~30–40 d postchallenge, the mice that received memory CD8 T cells from aged animals had fewer absolute numbers of DbNP396- or DbNP366-specific donor cells present in the spleen (Fig. 5*F*, 5*G*), lungs, and mediastinal and cervical lymph nodes (data not shown). Note that the tetramer$^-$ donor cells in Fig. 5*B* and 5*C* represent unrelated donor CD8 T cells transferred. In the

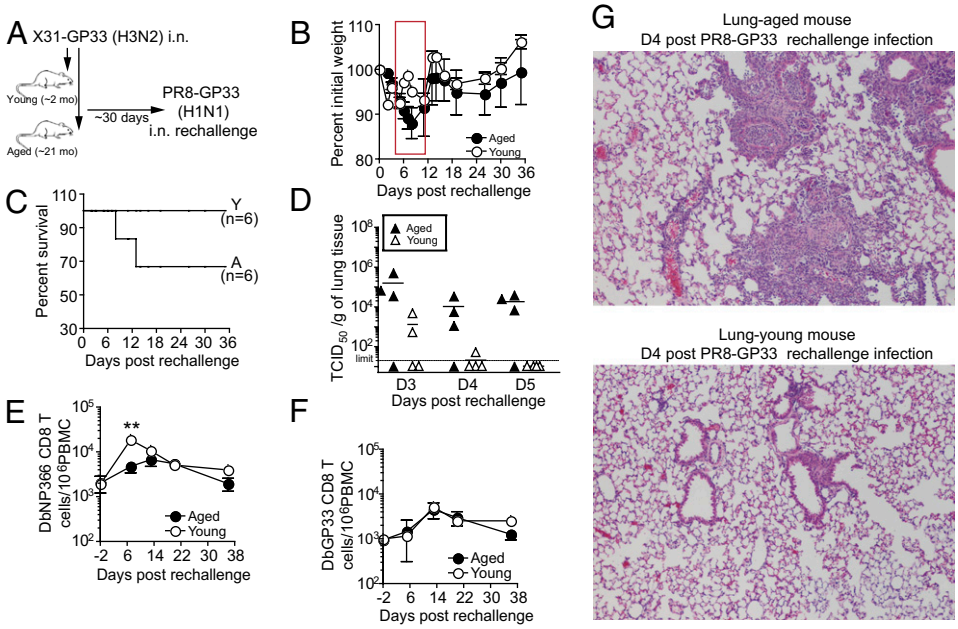

FIGURE 4. Heterosubtypic immunity to secondary influenza virus infection is decreased with age. Young and aged mice that had recovered from primary X31-GP33 infection were challenged with heterologous PR8-GP33 virus 33 d after primary infection (*A*), and weight loss (*B*) and mortality (*C*) were monitored. *D*, Viral titers in the lungs of aged (black) and young (white) mice post PR8-GP33 rechallenge infection (days 3–5 p.i.) were determined on Madin-Darby canine kidney cell monolayers. The frequency of both DbNP366-specific (*E*) and GP33-specific (*F*) memory CD8 T cells was monitored longitudinally in the PBMCs of individual aged (black symbols) and young (white symbols) mice. *G*, Lung pathology of formalin-fixed lung samples was determined at day 4 post PR8-GP33 rechallenge infection by H&E staining. Original magnification ×20. Data are representative of two to five independent experiments including 6–12 mice/experiment. $^{**}0.01 > p > 0.001$ by unpaired two-tailed *t* test. A, aged; Y, young.

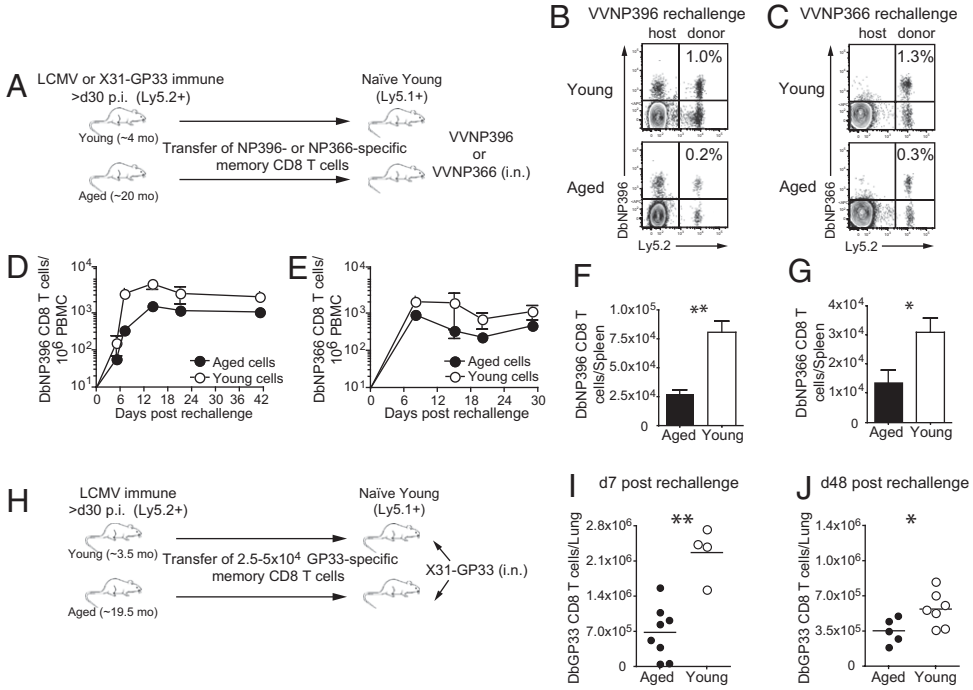

FIGURE 5. Memory CD8 T cells from aged mice are severely compromised in their ability to mount robust recall responses. CD8 T cells were purified from LCMV-immune (day 48) or influenza virus-immune (day 54, X31-GP33) young and aged Ly5.2$^+$ mice, and equal numbers of DbNP396 or DbNP366-specific memory CD8 T cells from young or aged mice were adoptively transferred to naive congenic (Ly5.1$^+$) mice. Recipient mice were then challenged i.n. with VVNP396 or VV expressing influenza virus NP366 epitope (*A*). Secondary responses of the donor (Ly5.2$^+$) DbNP396- and DbNP366-specific CD8 T cells were monitored in the blood (*B–E*) and tissues (*F*, *G*) following VV challenge. Lower frequencies (*B*, *C*) and absolute numbers (*D–G*) of virus-specific CD8 T cells derived from aged donors (black symbols/bars) were observed compared with their young counterparts (white symbols/bars). When sacrificed ~30–40 d post secondary infection, the mice that received memory CD8 T cells from aged animals (black bars) had fewer donor-derived tetramer$^+$ CD8 T cells present in the spleen (*F*, *G*). Plots in *B* and *C* were gated on CD8 T cells. Error bars (*D–G*) represent SEM. Data shown in *A–G* are representative of two to three independent experiments including 8–10 mice/experiment. CD8 T cells were purified from LCMV-immune (>day 30) young and aged Ly5.2$^+$ mice and equal numbers of DbGP33-specific memory CD8 T cells from young or aged mice were adoptively transferred to naive congenic (Ly5.1$^+$) mice. Recipient mice were challenged i.n. with X31-GP33 (*H*). At days 7 (*I*) and 48 (*J*) post rechallenge infection, the lungs of recipient mice were isolated, and the absolute numbers of donor (Ly5.2$^+$) DbGP33-specific CD8 T cells were examined. Lower absolute numbers of virus-specific donor CD8 T cells were observed in lungs of mice that received memory cells from aged donors (black symbols) compared with those that received memory cells from young donors (white symbols) (*I*, *J*). Error bars (*I*, *J*) represent SEM. Data shown in *H–J* are representative of two independent experiments including 10–12 mice/experiment. *$0.05 > p > 0.01$; **$0.01 > p > 0.001$ by unpaired two-tailed *t* test.

transferred donor pool, the NP396- or NP366-specific CD8 T cells represented 1–5% of CD8 T cells. Following challenge, the frequency of these cells among donor CD8 T cells increased to ~30–60% with memory CD8 T cells from young mice always expanding better than memory CD8 T cells from aged mice. It is unlikely that altered viral control explains these differences in CD8 T cell expansion because viral load in the lungs was similar on day 5 postchallenge in recipients of memory CD8 T cells from young versus aged mice (data not shown). It is also worth pointing out that the number of epitope-specific memory CD8 T cells adoptively transferred in these experiments is ~6–40-fold lower than the number required for substantial viral control in this model (>2–3 $\times$ 10^5 memory CD8 T cells) (43), further supporting the notion that the difference in proliferative expansion of memory CD8 T cells from aged versus young mice was not due to differences in control of the challenge infection.

These studies focused on secondary responses of the immunodominant CD8 T cell populations that were also the populations numerically reduced following primary infection. To examine whether these defects in proliferative capacity also extended to GP33-specific memory CD8 T cells and to examine the recall response following respiratory influenza virus infection, we designed an additional set of adoptive transfer experiments (Fig. 5*H*). In these studies, we also investigated whether the differences in the early secondary response in the blood shown in Fig. 5*B–E* reflected defects in the secondary response at the site of infection. Thus, equal numbers of Ly5.2$^+$ GP33-specific CD8 T cells from young or old LCMV-immune mice were adoptively transferred to young congenic (Ly5.1$^+$) recipient mice, and 1 d later, these mice were challenged i.n. with X31-GP33. On days 7 and 48 postchallenge, the number of GP33-specific CD8 T cells from young versus aged donors was determined in the lungs. As shown in Fig. 5*I*, significantly lower numbers of virus-specific CD8 T cells derived from aged compared with young donors were found in the lungs of recipient mice on day 7 p.i. This difference was sustained at later times postchallenge (~days 40–50) in the lungs (Fig. 5*J*) and spleens (data not shown) of recipient mice.

Decreased proliferative capacity of aged memory CD8 T cells upon secondary infections

The results described above suggested that memory CD8 T cells from aged mice have a reduced capacity to proliferate and expand during challenge infection compared with memory CD8 T cells from young mice. To address this issue further, mice were given BrdU during the secondary responses. Equal numbers of Ly5.2$^+$ NP396-specific CD8 T cells from young or old LCMV-immune mice were adoptively transferred to young congenic (Ly5.1$^+$) recipient mice, and 1 d later, these mice were challenged i.n. with VVNP396. On day 5.5 p.i., mice received i.p. BrdU injection and were sacrificed 12 h later. A second set was given BrdU on day 6.5

and analyzed on day 7 (Fig. 6*A*). On average, ~27% of young donor NP396-specific CD8 T cells incorporated BrdU in the lungs of recipient mice on day 6 post rechallenge (Fig. 6*B*, 6*D*, 6*F*). In contrast, BrdU incorporation was observed in only 14% of aged donor DbNP396-specific cells in the lungs of recipient mice at that same time. A similar trend was observed in lungs of recipient mice on day 7 postchallenge (Fig. 6*C*, 6*E*, 6*G*). As a result, the absolute number of BrdU$^+$ donor cells was substantially reduced in the lungs of aged compared with young mice on both days analyzed (Fig. 6*F*, 6*G*). Thus, reduced numbers of BrdU$^+$ cells following challenge infection suggest defects in cell division or survival of dividing cells and underlie the failure of memory CD8 T cells to expand vigorously upon reinfection.

These observations further confirm that aged memory cells bear cell-intrinsic defects that impair their ability to mount vigorous recall responses upon secondary infection and that these defects are apparent systemically and at the site of infection for multiple specificities as well as following different challenge infections.

Discussion

Studies have previously demonstrated that aged animals, like aged humans, are more susceptible to infection (33, 35–37). These studies have mainly focused on delayed and reduced effector T cell responses in aged mice during primary infections. Less information is available about memory CD8 T cells and especially responses to secondary infections during aging. In the current work, we found a substantial defect in the ability of memory CD8 T cells from aged mice to mount robust recall responses compared with memory CD8 T cells from young mice. This deficiency is of significance, considering that CD8 T cells are important mediators of control of intracellular infections, especially for many pathogens that affect the elderly such as influenza virus. In old mice, the defects in secondary T cell responses are severe and result in a delayed and impaired secondary effector CD8 T cell response. Upon challenge with a virulent and rapidly replicating and/or spreading pathogen such as influenza virus, the ability to quickly expand a pool of secondary effectors may be crucial to blunt the spread of infection. A delay in the speed of the T cell responses could not only result in greater virus dissemination and virus-induced pathology, but also will allow an increase in the number of infected cells and Ag burden. Once the sluggish aged T cells catch up, the increased Ag burden could lead to enhanced immunopathological damage. Thus, the defective and slow recall response of CD8 T cells from old mice could be damaging in two ways: by allowing increased early virus spread and then by contributing to immune mediated tissue damage in an effort to control the infection.

Although the precise reason for the poor recall responses of CD8 T cells from aged mice is not clear, several scenarios are worth considering. One possible reason for this difference is that the delayed control of primary infection in aged mice (33, 35–37 and data not shown) leads to prolonged exposure to Ag during priming. The duration and/or amount of stimulation by Ag during primary acute infection can alter the kinetics of memory T cell differentiation favoring effector memory-like T cells when Ag exposure is elevated or moderately prolonged (48, 49). A second possibility is that changes in the inflammatory environment, which are also known to impact memory T cell differentiation (50), occur during primary infection in aged versus young mice and that these changes lead to a delay or defect in the development of robust CD8 T cell memory in old mice. Either delayed viral clearance or an altered inflammatory environment would be consistent with our data demonstrating that fewer memory T cells from aged mice expressed CD62L, CD127, and CD27 compared with young mice, but future studies are necessary to investigate these issues in more depth. In addition, an altered TCR repertoire with age (19) could result in different levels of clonal competition

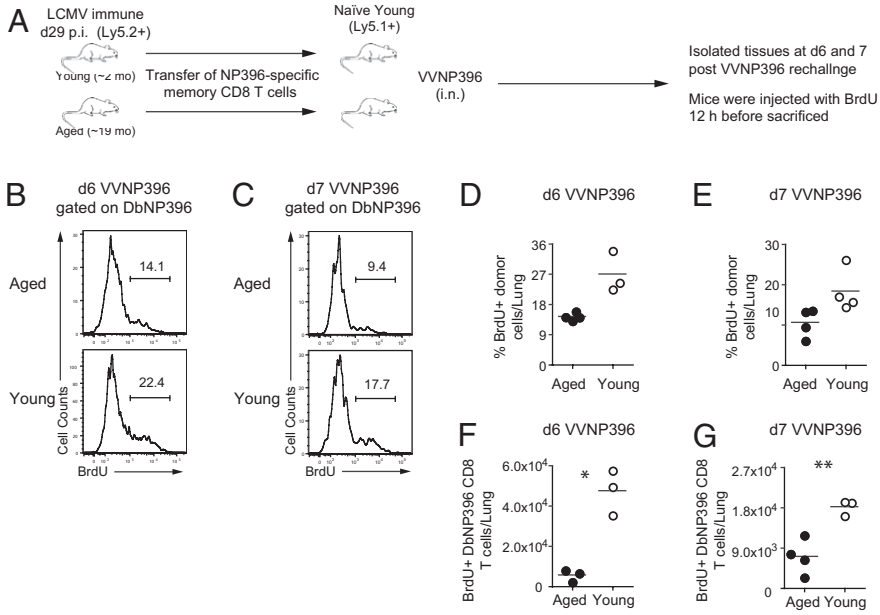

FIGURE 6. Memory CD8 T cells from aged mice had a decreased ability to proliferate upon secondary infections. CD8 T cells were purified from LCMV immune (day 29) young and aged Ly5.2$^+$ mice, and equal numbers of DbNP396-specific memory CD8 T cells from young or aged mice were adoptively transferred to naive congenic (Ly5.1$^+$) mice (*A*). Recipient mice were then challenged i.n. with VVNP396. Twelve hours preanalysis, mice were injected with BrdU i.p. (*B–G*). Secondary responses of the donor (Ly5.2$^+$) DbNP396-specific CD8 T cells were monitored in the lungs. Plots in *B* and *C* show representative histograms of BrdU staining in the lungs of young recipient mice at days 6 (*B*) and 7 (*C*) post VVNP396 rechallenge. Histograms are gated on donor NP396-specific CD8 T cells, and numbers indicate the percentages of BrdU$^+$ cells. The frequencies and numbers of BrdU$^+$ donor NP396-specific CD8 T cells derived from aged (black symbols) and young (white symbols) mice in the lungs of recipient mice at day 6 and 7 post VVNP396 rechallenge are shown in *D–G*, respectively. Data are representative of two independent experiments including three to four mice/experiment. *$0.05 > p > 0.01$; **$0.01 > p > 0.001$ by unpaired two-tailed *t* test.

during priming, changing the rate of differentiation or proportion of different memory T cell subsets. Finally, other cell-intrinsic or external factors such as inherent T cell senescence (51–53) or Tregs (23, 24) could influence the initial development of T cell memory in aged mice. It is possible that more than one of these mechanisms contributes to changes in CD8 T cell memory with age. Whichever mechanism(s) contribute, the current experiments indicate that, once established, major aspects of the defective recall responses in CD8 T cell memory from aged mice are cell-intrinsic. The reduced numbers of $BrdU^+$ aged memory CD8 T cells following challenge infection suggest defects in cell division and/or survival of dividing cells. It is also possible that aged memory CD8 T cells do not get access to secondary lymphoid organs or dendritic cells as well as young memory CD8 T cells. Whether due to migration, proliferative potential, or other factors, the defective secondary responses of memory CD8 T cells from aged mice were cell-intrinsic and could have important implications for vaccinating the elderly. Some of the differences we observed between memory CD8 T cells from young and aged mice in their recall capacity and protective immunity could also reflect differences between effector memory and central memory T cells (43). The differences in production of cytokines by memory CD8 T cells from old mice following LCMV versus influenza virus infection are also intriguing. The reason the LCMV-induced memory CD8 T cells in old mice are less functional is not entirely clear, but might reflect the greater propensity of LCMV to persist in the mouse and induce T cell dysfunction when primary immune responses are suboptimal. However, future studies are necessary to fully examine the scope of changes and pathways involved in the severe defects in memory T cells generated in aged mice following viral infections.

Age-related changes in organismal physiology or lifespan are often related to cellular senescence, changes in telomere biology, genes involved in endocrine signaling, stress responses, or metabolism (54). It remains unclear how these pathways influence the responsiveness of memory lymphocytes in aged mice or humans. However, understanding of age-related changes in T cell function and response to infection is a central issue in developing strategies to improve immunity in the elderly. Dissecting the mechanisms of the changes in immunity with age will also contribute to the development of more effective vaccines for elderly humans. The studies described in this paper demonstrate that the defects intrinsic to aged CD8 T cells are at least partially responsible for diminished immunity of aged mice. Our studies indicate that strategies that overcome proliferative deficiencies may prove beneficial, though efforts designed to enhance the vigor of CD8 T cells in the elderly should be carefully balanced with the potential to cause immunopathological damage. Future studies should focus on a better understanding of how these pathways operate in an aged host and should provide opportunities for therapeutics or improved vaccines in the elderly.

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Disclosures

The authors have no financial conflicts of interest.

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