Age-associated decline in T cell repertoire diversity leads to holes in the repertoire and impaired immunity to influenza virus

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A diverse T cell repertoire is essential for a vigorous immune response to new infections, and decreasing repertoire diversity has been implicated in the age-associated decline in CD8 T cell immunity. In this study, using the well-characterized mouse influenza virus model, we show that although comparable numbers of CD8 T cells are elicited in the lung and lung airways of young and aged mice after de novo infection, a majority of aged mice exhibit profound shifts in epitope immunodominance and restricted diversity in the TCR repertoire of responding cells. A preferential decline in reactivity to viral epitopes with a low naive precursor frequency was observed, in some cases leading to "holes" in the T cell repertoire. These effects were also seen in young thymectomized mice, consistent with the role of the thymus in maintaining naive repertoire diversity. Furthermore, a decline in repertoire diversity generally correlated with impaired responses to heterosubtypic challenge. This study formally demonstrates in a mouse infection model that naturally occurring contraction of the naive T cell repertoire can result in impaired CD8 T cell responses to known immunodominant epitopes and decline in heterosubtypic immunity. These observations have important implications for the design of vaccine strategies for the elderly.

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Abbreviations used: BAL, bronchoalveolar lavage; NP, $NP_{366-374}/D^b$; PA, $PA_{224-233}/D^b$.

It has been well-established that immunity declines with aging (1–5). The elderly are more susceptible to infections, particularly to those caused by newly emerging and reemerging pathogens, and such infections are often of greater severity. In addition, vaccines are considerably less effective in the elderly (6–10). Whereas the function of aged CD4 T cells has been extensively investigated and distinct defects defined, the impact of aging on CD8 T cell function is poorly understood (1, 3–5, 11, 12). It has been suggested that apparent declines in CD8 T cell effector function may instead be the consequence of age-associated changes in the composition of the CD8 T cell pool (13), consistent with reports that naive CD8 T cells from aged mice are fully functional (14, 15).

The ability of individuals to generate effective T cell responses to newly encountered infections and to respond to vaccination requires the maintenance of a diverse repertoire of T cells (16–18). Thus, it has been speculated that declining T cell repertoire diversity associated with aging is a contributing factor to the impaired ability of aged individuals to mount effective immune responses to infections and vaccines (1, 5, 19–23). The functional diversity of the αβ TCR repertoire in the spleens of young mice has been estimated to be $\sim 2 \times 10^6$ clones, with ~10 cells per clone (24). However, several age-associated changes are thought to lead to reductions in both the size and diversity of the naive T cell repertoire. Fewer T cells are produced in the thymus, leading to reduced numbers of naive T cells in the periphery (25). The naive T cell repertoire also becomes increasingly constrained by the progressive accumulation of peripheral T cells exhibiting a memory phenotype, believed to be the result of the accumulated antigen experience of the individual (13, 20, 26). The diversity of the memory repertoire is further compromised by the development of age-associated CD8 T cell clonal expansions, which can comprise 70–80% or more of the total CD8 T cell compartment in some aged animals (27–30). Collectively, declining numbers and diversity of naive T cells emerging from the aged thymus, progressive increase in the proportion of antigen-experienced compared with naive T cells, and the

development of large clonal expansions result in substantially reduced diversity among CD8 T cells in aged mice.

Significant progress has been made in dissecting the diversity of the CD8 T cell response to several pathogens. For example, the repertoire of CD8 T cells responding to two immunodominant influenza virus epitopes, $NP_{366-374}/D^b$ (NP), and $PA_{224-233}/D^b$ (PA), in C57BL/6 mice after primary influenza virus infection has been well characterized, and the responses to NP and PA are dominated by CD8 T cells expressing Vβ8.3 and Vβ7 elements in their TCRs, respectively (31–36). Whereas the repertoire of $V\beta8.3^+$ T cells specific for NP is limited and highly conserved between individual mice (termed a public repertoire), the repertoire of $V\beta7^+$ T cells specific for PA is extremely diverse and varies among individual mice (termed a private repertoire) (36–38). The limited repertoire diversity of NP- compared with PA-specific T cells predicts a lower number of clonotypes specific for NP compared with PA. Indeed, it has been estimated that an individual mouse contains NP-specific $V\beta8.3^+$ T cells bearing a mean of 8 different clonotypes, 3 of which are shared (public), whereas each mouse contains 20 different $V\beta7^+$ clonotypes specific for PA, none of which are shared (private) (39).

In this study, we take advantage of the well-characterized influenza virus model to study the impact of aging on the diversity of the antiviral CD8 T cell response. We show by in vivo limiting dilution analysis that the functional precursor frequency of CD8 T cells for NP in young naive mice is ~10-fold smaller than that for PA. We further observed a preferential decline, and in some cases a loss, of the NP-specific T cell responses in aged mice infected de novo with influenza virus. The decline in NP responsiveness in aged mice was associated with perturbations in the repertoire of NP-specific cells which were variable in individual mice. Furthermore, reduced NP reactivity in aged mice generally correlated with an impaired ability to control virus after heterosubtypic secondary challenge. These data directly demonstrate the impact of an age-associated decline in T cell repertoire diversity on the capacity to respond to newly encountered antigens. Importantly, the data show that the age-associated decline in CD8 T cell repertoire diversity can be so profound for responses with low naive precursor frequencies as to result in the development of "holes" in the repertoire for normally immunodominant epitopes, and may lead to compromised protective immunity.

RESULTS

In vivo limiting dilution analysis reveals a reduced functional precursor frequency of NP- compared with PA-specific T cells

To determine the numbers of functional T cell precursors in young C57BL/6 mice specific for NP and PA, and also a third major epitope, $PB1_{703-711}/K^b$ (PB1), we performed an in vivo limiting dilution analysis. Graded numbers of CD8 T cells from young C57BL/6 mice ranging from 3×10^6 to 3×10^4 were transferred into T cell–deficient ($TCR\beta\delta^{-/-}$ mice) hosts just before intranasal infection with influenza virus. Naive precursor frequencies are not readily detectable by tetramer analysis, so the ability of precursors to proliferate and expand in number after viral infection such that they could be measured by tetramers defined them as functional precursors. The magnitude of the donor T cell response to the three epitopes was determined on day 14 after infection by tetramer staining. Because the recipient is genetically devoid of all T cells, the epitope-specific CD8 T cell responses in these mice are mediated by the adoptively transferred donor CD8 T cells. Representative staining data showing the T cell response to NP, PA, and PB1 detected in individual recipient mice are shown in Fig. 1, and cumulative data showing the ratio of T cells specific for NP/PA/PB1 from multiple individual recipients are presented in Fig. 2. The data show that a CD8 T cell response to PA could be detected when as few as 10^5 donor CD8 T cells were transferred, whereas a

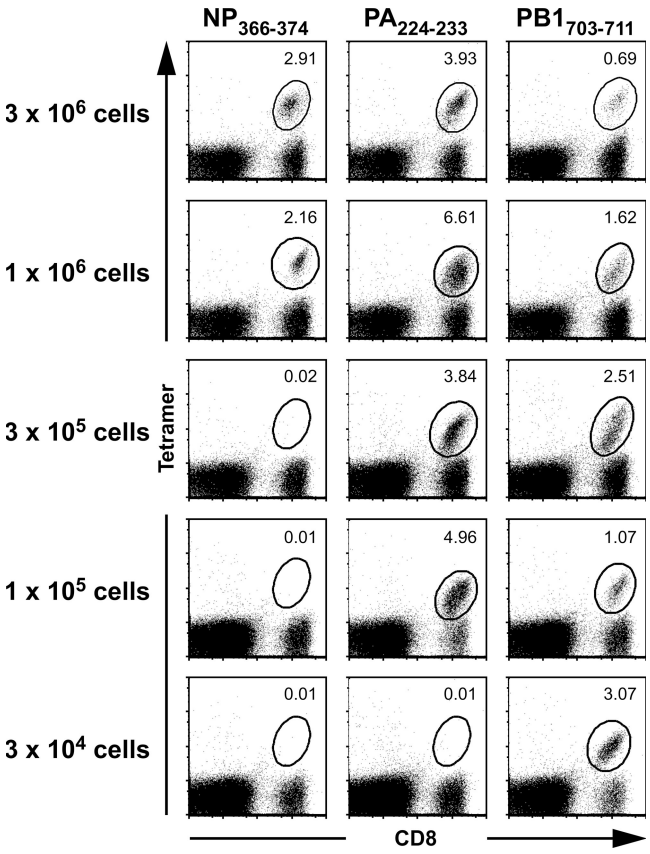

Figure 1. In vivo limiting dilution analysis to determine functional precursor frequencies of NP-, PA-, and PB1-specific CD8 T cells. The indicated numbers of CD8 T cells isolated from young naive C57BL/6 donor mice were adoptively transferred into young $TCR\beta/\delta^{-/-}$ recipient mice 1 d before infection with influenza virus. Lung tissue was harvested from individual recipient mice 14 d after infection. Each FACS dot plot shows the frequency of donor lymphocytes present within the lung tissue of individual recipient mice that stained positive for both CD8 and either influenza NP ($NP_{366-374}/D^b$), PA ($PA_{224-233}/D^b$), or PB1 ($PB1_{703-711}/K^b$) MHC class I tetramer. The data are representative examples of analysis of 3–4 mice per dilution, shown in Fig. 2.

robust and consistent response to NP could only be detected in recipient mice that received 10^6 or more cells, suggesting a 10-fold difference in the functional precursor frequency for these two epitopes. The pool of precursors was largest for the PB1 epitope, as a response to this epitope was detected after transfer of as few as 3×10^4 cells. Thus, there is an approximate 1:10:30 ratio of functional T cell precursors for the NP, PA, and PB1 epitopes, respectively, in young C57BL/6 mice.

Preferential age-associated loss in responsiveness to NP

We next investigated the response of individual aged mice to the NP, PA, and PB1 epitopes after primary influenza virus infection. Our prediction was that the relatively low frequency of NP precursors would be further reduced by age-associated contraction of the naive T cell repertoire, resulting in a preferential loss of reactivity to the NP epitope in aged mice. Before the analysis, we prescreened peripheral blood lymphocytes from aged mice and excluded those mice with evidence of large T cell clonal expansions from the study to both eliminate any impact of clonal expansions on the overall diversity of the repertoire and allow for a more straightforward interpretation of the data. We intranasally infected individual young (6–12-wk-old) and aged (>18-mo-old) naive mice with influenza virus, and analyzed the response generated 10 d after infection. Analysis of total CD8 T cells in the bronchoalveolar lavage (BAL) and lung tissue revealed that comparable frequencies and absolute numbers of CD8 T cells were elicited in young and aged infected mice (Fig. 3, A and B), arguing against a major shift in kinetics in this analysis. However, analyses of the epitope specificities of the CD8 T cells elicited in aged mice revealed major perturbations in the composition of the antiviral response in the majority of mice (Fig. 3, C and D). The most striking effect was an approximately fivefold reduction in the CD8 T cell response to NP in aged mice compared with young mice, both in terms of the median frequencies (2.39% versus 18.20%) and absolute numbers (5,110 cells versus 44,429 cells) of NP-specific CD8 T cells detected in the lung airways. These data are in agreement with a previous study showing a reduced NP response in aged mice after primary influenza virus infection (12). There were, however, 3 aged mice among the 20 analyzed that exhibited an NP-specific response that was comparable in magnitude to that characteristic of a young mouse. In contrast, the median percentage of CD8 T cells responding to the PA epitope was not significantly different in the groups of young and aged mice examined, although some individual aged mice showed dramatic reductions in response to this epitope. The mean frequencies and absolute numbers of PB1-specific CD8 T cells were also not statistically different in aged compared with young mice, but many aged mice showed an elevated response to PB1. In addition, analysis at 14 d after infection showed the same relative ratios of epitope-specific responses (unpublished data), arguing against a simple shift in kinetics as an explanation for the data. Collectively, these results demonstrate a correlation between the ability of aged C57BL/6 mice to mount CD8 T cell

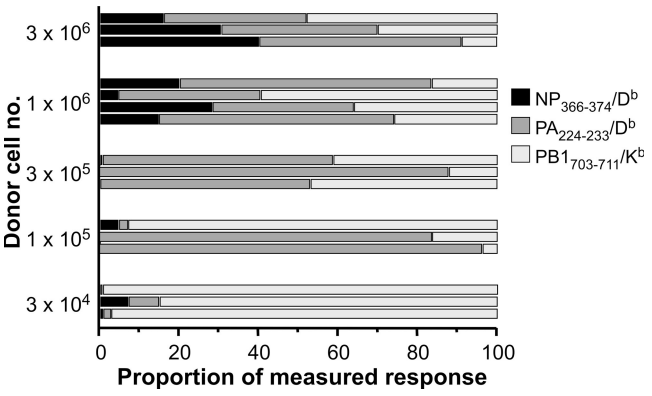

Figure 2. The functional precursor frequency of CD8 T cells in young C57BL/6 mice is lower for NP than for PA or PB1. The indicated numbers of purified CD8 T cells isolated from young C57BL/6 mice were adoptively transferred into young TCR $\beta/\delta^{-/-}$ recipient mice via i.v. injection 1 d before infection with influenza virus. Donor CD8 T cells specific for NP ($NP_{366-374}/D^b$), PA ($PA_{224-233}/D^b$), or PB1 ($PB1_{703-711}/K^b$) present in lung tissue of individual mice on day 14 after infection were identified by flow cytometric analyses. The relative proportion of total measured donor cell response was calculated by dividing the percentage of each epitope-specific CD8 T cell population by the total percentage of CD8 T cells specific for all three epitopes examined (e.g., percentage of NP/[percentage of NP + percentage of PA + percentage of PB1]). Each bar represents data calculated from an individual infected recipient mouse.

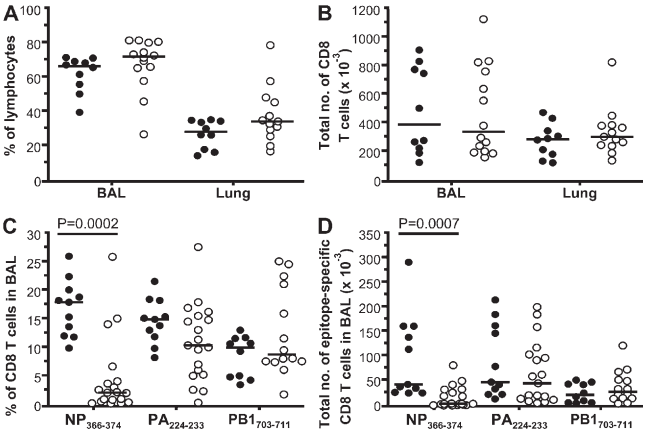

Figure 3. A majority of aged C57BL/6 mice show reduced CD8 T cell responses to influenza NP, but not to PA or PB1. BAL and lung tissue were harvested from individual young (closed symbols) and aged (open symbols) C57BL/6 mice 10 d after influenza virus infection. Young and aged mice presented with comparable frequencies (A) and absolute numbers (B) of CD8 T cells within the BAL and lung tissue. The frequencies (C) and total numbers (D) of CD8 T cells positive for NP ($NP_{366-374}/D^b$), PA ($PA_{224-233}/D^b$), or PB1 ($PB1_{703-711}/K^b$), assessed by tetramer staining of the BAL cells from infected mice, are shown. Bars indicate the medians calculated from data compiled from four independent experiments (11 young and 19 aged mice for NP and PA, and 10 young and 14 aged mice for PB1). Significance was assessed using the Mann-Whitney rank test (two-tailed, 95% confidence).

responses to immunodominant influenza virus epitopes and the relative precursor frequencies for each epitope in young mice (Figs. 1 and 2).

One possible explanation for the dramatic loss of NP responsiveness in aged mice could be epitope-mediated suppression, as it has previously been shown that the response to NP can be suppressed by the presence of a large PA response (40). To rule out this possibility, we infected young and aged mice with a mutant influenza virus that had been modified by site-directed mutagenesis to generate a variant PA peptide that can no longer bind to D^b (41, 42). The data in Fig. 4 show that even in the absence of the PA epitope, there was virtually no response to NP in the majority of infected aged mice, reinforcing the notion that the absence of the NP response was predominantly caused by an age-associated decline in NP precursor frequency rather than PA-mediated suppression.

The TCR Vβ repertoire is perturbed in aged mice

To further characterize the impact of aging on the response to NP and PA in influenza virus-infected mice, we analyzed the Vβ usage of CD8 T cells responding to each of the epitopes in individual mice. We determined the percentage of CD8 T cells responding to NP, PA, and PB1 epitopes in a new cohort of young and aged mice by tetramer analysis, and then analyzed the Vβ usage of the tetramer-positive cells using a limited panel of Vβ-specific antibodies (Vβ2, Vβ4, Vβ5.1 + 5.2, Vβ7, Vβ8.3, and Vβ13). Individual mice in Fig. 5 are represented by unique symbols, such that the epitope reactivity (left) and the TCR Vβ usage (right) can be correlated in each mouse.

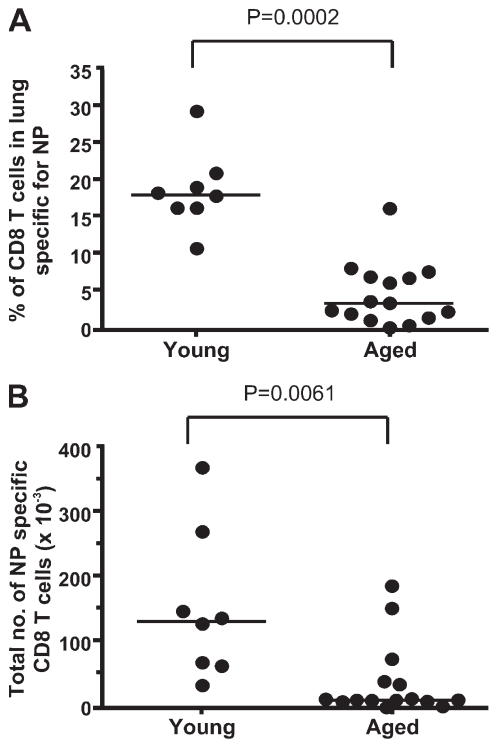

Figure 4. Aged C57BL/6 mice show reduced NP responses when infected with an influenza virus lacking the PA epitope. Young and aged C57BL/6 mice were intranasally infected with PA-deficient influenza virus (600 EID_{50}) and lung tissue was harvested 10 d later. The frequencies (A) and total numbers (B) of CD8 T cells positive for NP ($NP_{366-374}/D^b$) in the lungs of individual infected mice are shown. Bars indicate the medians calculated from data compiled from two independent experiments (total of 8 young and 15 aged mice). Significance was assessed using the Mann-Whitney rank test (two-tailed, 95% confidence).

Analysis of the NP-specific repertoire (Fig. 5 A) showed that, consistent with previous observations (32, 36), there was a strong bias toward T cells expressing a $V\beta8.3^+$ TCR in the response to NP among young mice, with 45–65% of the responding T cells being $V\beta8.3^+$. However, there was individual variation in the repertoire of NP-specific cells. For example, two of the young mice showed a major $V\beta5^+$ component of the response. In contrast, and consistent with the data in Fig. 3, analysis of aged mice showed that only two out of five mice retained substantial recognition of the NP epitope. Importantly, the Vβ usage among the NP-specific T cells of these individual aged mice showed a different distribution from that in young mice in that the percentage of T cells bearing $V\beta8.3^+$ receptors were low in the two aged mice that retained a substantial NP response (open circle and diamond). In contrast, two of three mice with a weak response to NP predominantly used a $V\beta8.3^+$ TCR, although analysis with Vβ-specific antibodies did not allow us to determine whether this was the Vβ8.3Jβ2.2 sequence shown to dominate the response to NP in virtually all young mice (36). In another aged mouse (open squares), $V\beta2^+$ T cells were dominant among the few NP-specific cells detected.

A similar analysis of the PA-specific T cell response revealed a different pattern (Fig. 5 B). Although there was a modest reduction in the frequency and numbers of PA-specific T cells elicited in aged compared with young mice, there was no evidence for perturbation in the TCR repertoire of responding T cells. All young and aged infected mice analyzed expressed $V\beta7^+$ receptors, which were previously shown to dominate the response to PA in young mice (38). Finally, the response to PB1 was enhanced in three out of five aged mice (Fig. 5 C). Although none of the antibodies used revealed a dominant Vβ usage in the young mice, one aged mouse had an elevated frequency of $V\beta13^+$ T cells in PB1-specific cells.

To analyze the repertoires of CD8 T cells responding to NP and PA epitopes in greater detail than was possible with the use of Vβ antibodies, we performed spectratype analysis on sorted populations of NP- and PA-specific CD8 T cells from a separate cohort of individual aged and young mice 10 d after influenza virus infection to determine CDR3 length diversity. We used 22 Vβ primers to span virtually the entire TCR β-chain repertoire, with the exception of Vβ17 and Vβ19, which are pseudogenes in C57BL/6 mice (43–45). Representative spectratype data from naive young mice and NP- and PA-specific cells isolated from one young and two aged influenza virus-infected

mice are shown in Fig. 6, and the numbers of peaks within each of the 22 Vβ spectrotypes are summarized in Table I for NP-specific T cells and Table II for PA-specific T cells. The spectratype analysis of CD8 T cells from young naive mice revealed the expected Gaussian profiles indicative of a diverse, naive repertoire (Fig. 6, top line) (46). In contrast, analyses of purified populations of epitope-specific T cells isolated from individual influenza virus–infected young and aged mice revealed the presence of distinct peaks representing one or a limited number of CDR3 sizes, indicative of expanded populations of NP- or PA-specific T cells expressing a particular TCR Vβ element and CDR3 size (Fig. 6, lines 2–7). Analysis of the repertoire of NP- and PA-specific T cells for young mice summarized in Tables I and II, respectively, represents the first assessment of the entire Vβ repertoire for NP and PA in individual mice, and makes the important point that despite the dominant Vβ8.3 usage for NP and Vβ7 usage for PA, the T cell repertoire in young mice is very diverse and includes T cells expressing 8–15 Vβs for NP and 12–18 Vβs for PA. In addition to the well-described dominance of Vβ8.3 for NP and Vβ7 for PA, other Vβs make an important contribution to the responses, as we found there were 5 additional Vβs for NP (Vβ1, 4, 7, 8.2, and 15) and 3 additional Vβs for PA (Vβ5.2, 6, and 11) that were expressed in all individual young mice examined.

The data revealed several prominent features regarding repertoire perturbations associated with aging. First, consistent with the Vβ antibody analysis performed on a separate cohort of young and aged mice (Fig. 5), the spectratype analysis revealed that Vβ8.3$^+$ T cells were represented in the repertoire of NP-specific T cells in all young mice, but in only three of six aged mice. Furthermore, consistent with exhaustive sequence data defining the public (shared between mice) nature of the Vβ8.3$^+$ repertoire of T cells specific for NP (36), the peaks from each individual young mouse, and the 3 of 6 aged mice that contained Vβ8.3$^+$ NP-specific T cells, all corresponded to a CDR3 length of 9 aa (unpublished data). In contrast, and again as predicted by our analyses using TCR Vβ-specific antibodies (Fig. 5), the dominant Vβ7 response found universally in young mice (38) was also represented in all aged mice (Fig. 6, lines 5–7, and Table II). In addition, Vβ6$^+$ PA-specific T cells were present in all young and aged mice analyzed.

Second, spectratype analysis of T cell receptor Vβ usage in individual aged mice highlights the important point that the aged repertoire is generally a subset of that present in young mice. For example, NP$^+$ cells from all young but only some aged mice expressed Vβ1, 4, 7, 8.2, 8.3, and 15. Similarly, PA$^+$ cells from all young but only some aged mice expressed Vβ5.2 and 11.

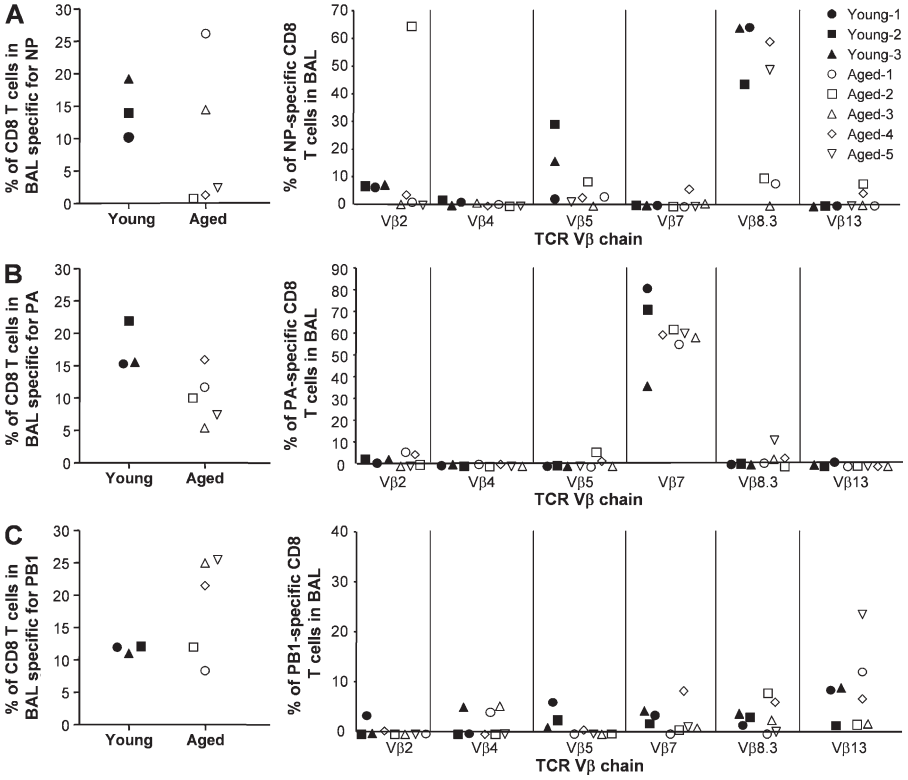

Figure 5. NP-specific CD8 T cells from aged mice exhibit altered Vβ usage. Young and aged C57BL/6 were intranasally infected with influenza virus and BAL was harvested on day 10 after infection. Panels on the left indicate the frequencies of CD8 T cells detected in the BAL of each individual mouse specific for the (A) NP ($NP_{366-374}/D^b$), (B) PA ($PA_{224-233}/D^b$), or (C) PB1 ($PB1_{703-711}/K^b$) epitopes. Panels on the right indicate TCR Vβ chain usage of epitope-specific CD8 T cells determined using the indicated anti-Vβ antibodies. Each symbol identifies an individual mouse. Closed symbols represent individual young mice ($n = 3$), and open symbols represent individual aged mice ($n = 5$).

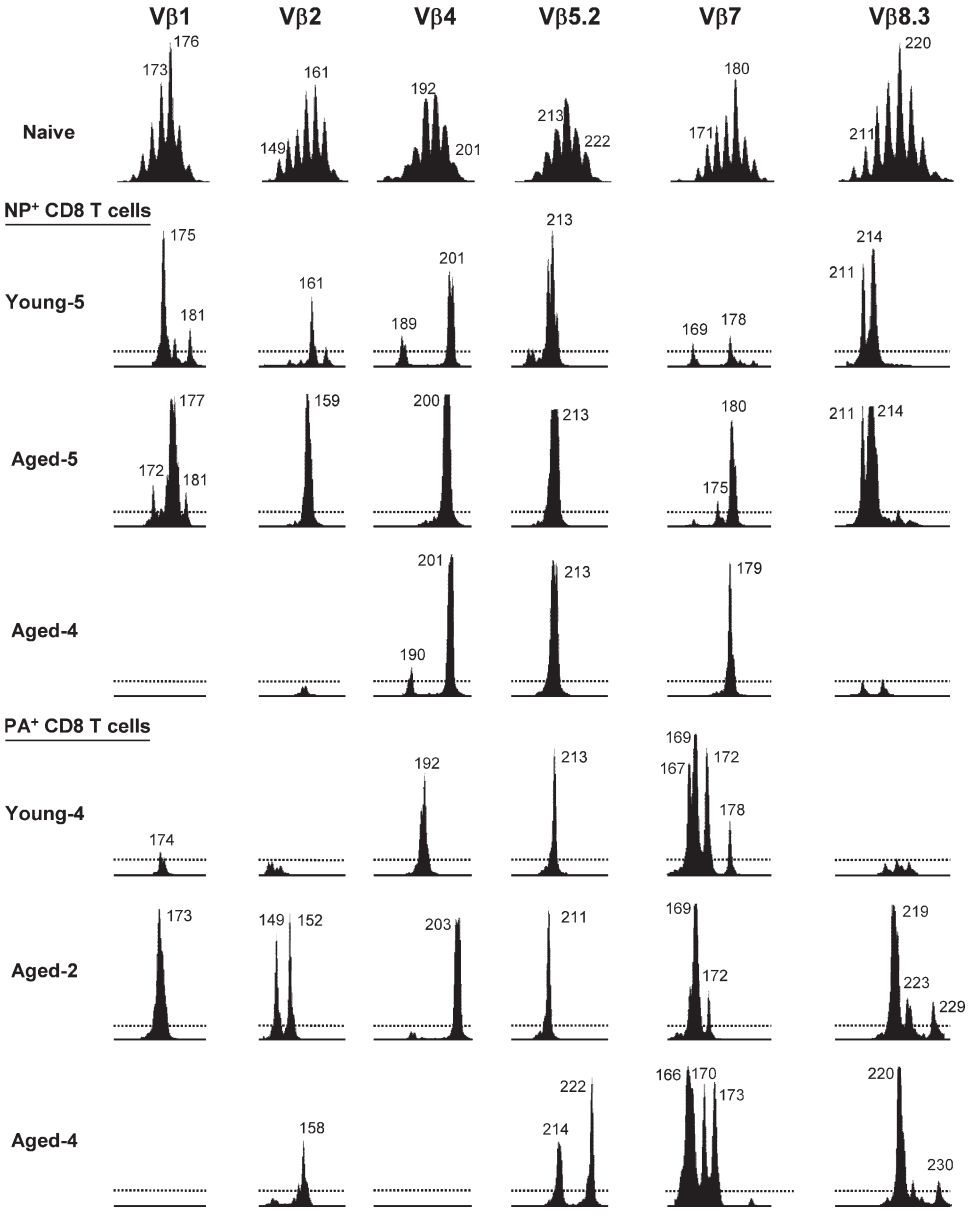

Figure 6. Spectratype profiles for selected Vβ gene families of NP- and PA-specific CD8 T cells from young and aged influenza virus–infected mice. Representative spectratype analysis of CD8 T cells from young, naive mice (top row) or purified NP- (middle rows) and PA-specific (bottom rows) T cells isolated by FACS-sorting from representative individual young and aged mice 10 d after influenza virus infection. Relative intensity is plotted along the y axis and nucleotide size is plotted along the x axis. The numbers refer to the size, in base pairs, of the individual expanded peaks. Young mouse 5, aged mouse 5, and aged mouse 4 had 28, 8, and 1% NP-specific cells among total CD8 T cells. Young mouse 4, aged mouse 2, and aged mouse 4 had 9, 19, and 4% PA-specific cells among total CD8 T cells, respectively. The complete spectratype analysis for all mice examined is presented in Tables I and II. The y axis is the same for all spectratype profiles, with the maximal height at 6,400 arbitrary units.

Third, there are also spectratype data that suggest that the aged repertoire contains unique responses that are not characteristic of the young repertoire. For NP, there was only 1 example in which new specificities at the Vβ level emerged (aged mouse #1 used TCR bearing Vβ18 and 20 in its response, which were not observed in the 5 young mice examined). In addition, in two separate cases (Vβ6 and 8.1) unique size bands emerged in aged mice within the Vβ spectratype (unpublished data). The observation of new responses emerging in aged mice was even more apparent in the PA response, which may be expected, as this is a more heterogeneous, promiscuous response. Thus, in individual aged mice, Vβ2, 8.2, 14, and 18 were a component of the response of aged, but not young mice. However, there was no Vβ specificity unique to aged mice that was shared among all aged individuals, which is consistent with the stochastic nature of the compensatory response.

Table I. Summary of spectratype analysis of purified NP-specific T cells[a]

Mouse number	T cell receptor Vβ[b]																					
	1	2	3.1	4	5.1	5.2	5.3	6	7	8.1	8.2	8.3	9	10	11	12	13	14	15	16	18	20
Young																						
Y1	1[c]	1	2	1	–	–	–	2	3	–	1	1	1	1	1	2	–	1	1	–	–	–
Y2	1	–	–	1	–	–	–	1	2	–	2	2	–	1	–	–	–	–	1	–	–	–
Y3	1	1	2	1	–	1	–	1	1	–	1	1	–	3	–	1	–	–	2	3	–	–
Y4	2	1	4	2	–	2	–	–	2	2	1	1	3	–	–	4	–	–	2	3	–	–
Y5	3	1	1	2	–	1	–	2	2	1	2	2	–	3	1	1	–	–	2	2	–	–
Aged																						
A1	1	–	–	1	–	–	–	1	–	–	1	1	–	1	–	–	–	–	1	1	1	1
A2	1	–	–	1	–	1	–	1	–	–	1	1	1	1	2	2	–	1	1	1	–	–
A3	–	–	–	–	–	1	–	1	1	–	–	–	–	–	–	1	–	–	–	–	–	–
A4	–	–	–	2	–	1	–	–	1	–	1	–	–	–	–	–	–	–	–	–	–	–
A5	3	1	–	1	–	1	–	2	2	1	1	2	2	2	2	–	–	2	–	1	–	–
A6	–	–	–	–	–	1	–	–	1	–	–	1	–	–	–	2	–	–	–	–	–	–

[a]NP tetramer+ T cells were sorted from individual young (2–3 mo) and aged (18 mo) mice 10 d after influenza virus infection. The percentage of NP+ cells for young mice ranged between 15 and 30%, and for individual aged mice numbers 1–6 the percentages were 5.6, 6.3, 8.2. 1.1, 8.1, and 8.2%, respectively.
[b]Spectratype analysis was carried out with a panel of 5′ Vβ and 3′ Cβ primers.
[c]Numbers of expanded peaks (>10% of the maximal peak height) for each Vβ-Cβ.

Collectively, analysis of the TCR repertoires for both NP and PA using Vβ-specific antibodies and CDR3 spectratype analysis revealed that the repertoires were dramatically impacted by aging. However, consistent with the lower precursor frequency and more limited repertoire diversity characteristic of NP-specific cells in young mice, the age-associated repertoire contraction had more profound implications for the NP-specific response.

Thymectomy results in preferential loss of NP reactivity

The data thus far suggest that the dramatic impact of aging on the CD8 T cell response to influenza virus is caused by declining repertoire diversity. As an independent confirmation, we examined the effect of thymectomy on the capacity of young mice to generate a CD8 T cell response against influenza virus. It has been shown that the age-associated involution of the thymus results in a decline in the numbers, and presumably the diversity, of the naive T cell pool caused by a reduced output of new thymic immigrants. Thymectomy of young mice mimics this effect by eliminating the production of new naive T cells. Thus, we hypothesized that influenza virus–infected thymectomized mice would mirror aged mice in their reduced ability to respond to NP. Young C57BL/6 mice were thymectomized at 5 wk of age, rested for 7 mo,

Table II. Summary of spectratype analysis of purified PA-specific T cells[a]

Mouse number	T cell receptor Vβ[b]																					
	1	2	3.1	4	5.1	5.2	5.3	6	7	8.1	8.2	8.3	9	10	11	12	13	14	15	16	18	20
Young																						
Y1	–	–	–	1[c]	–	1	–	2	1	–	–	4	–	–	1	2	–	–	–	–	–	–
Y2	–	–	1	–	–	2	–	1	3	–	–	–	–	1	2	–	–	–	1	1	–	1
Y3	–	–	–	1	–	1	–	2	3	–	–	–	1	–	1	1	1	–	–	1	–	–
Y4	1	–	–	1	–	1	–	1	4	2	–	–	2	1	1	1	–	–	1	2	–	–
Aged																						
A1	1	1	–	1	–	4	–	1	4	1	–	–	1	2	1	1	–	1	2	1	1	1
A2	1	2	–	1	–	1	–	1	2	2	1	4	4	1	1	2	–	1	2	1	–	–
A3	–	–	–	–	–	–	–	1	1	–	1	3	1	–	1	–	–	–	1	–	–	–
A4	–	1	–	–	–	2	–	2	3	–	1	3	1	1	1	–	1	–	–	1	1	–
A5	2	1	1	3	–	–	–	1	2	1	–	–	1	1	1	2	–	1	1	2	–	–
A6	–	–	–	2	–	–	–	3	3	–	–	–	2	–	–	1	–	–	–	2	–	–

[a]PA tetramer+ CD8 T cells were sorted from individual young (2–3 mo) and aged (18 mo) mice 10 d after influenza virus infection. The percentage of PA+ cells for young mice ranged between 8 and 30%, and for individual aged mice numbers 1–6 the percentages were 9.8, 19.4, 2.8. 4.4, 6.4, and 6.3%, respectively.
[b]Spectratype analysis was carried out with a panel of 5′ Vβ and 3′ Cβ primers.
[c]Numbers of expanded peaks (>10% of the maximal peak height) for each Vβ-Cβ.

and then infected with influenza virus. Thymectomy did not impact the ability of mice to respond to influenza virus infection, as similar frequencies and numbers of CD8 T cells were elicited in the lungs at 11 d after infection in thymectomized and control mice (Fig. 7, A and B). However, when compared with control age-matched animals, greatly reduced frequencies and numbers of CD8 T cells specific for NP were detected in the lungs of thymectomized mice at the peak of the immune response (Fig. 7, C and D). Thymectomy resulted in an ~7-fold reduction in the median frequency, and an ~10-fold reduction in median absolute number, of NP-specific CD8 T cells in the lungs of mice infected and analyzed at 7 mo after surgery. The impact of thymectomy on the NP-specific response appeared to be stronger and more consistent than that which occurs naturally with aging, as five out of five thymectomized mice generated significantly reduced frequencies and numbers of NP-specific T cells. In contrast, with one exception, the mean frequencies and numbers of PA- and PB1-specific CD8 T cells detected in the lungs of thymectomized mice were comparable to the control mice. One individual thymectomized mouse, indicated by the open circles, failed to respond to any of the three epitopes assayed, although this mouse appeared capable of mounting an antiviral CD8 T cell response, as indicated by the fact that there were comparable frequencies and numbers of CD8 T cells detected in the lung of this mouse (Fig. 7, A and B). This result suggests that this mouse was mediating an antiviral CD8 T cell response directed toward epitopes other than NP, PA, or PB1. Thus, in mice thymectomized when young, the effects of naive T cell repertoire contraction on the CD8 T cell response against influenza virus infection were enhanced and accelerated, in that 7-mo-old thymectomized mice consistently demonstrated a loss in responsiveness to NP normally seen in only a subset of 18-mo-old nonthymectomized mice (Figs. 3 and 5). This observation strengthens the link between reduced repertoire diversity and the compromised responsiveness to the normally immunodominant influenza virus NP epitope in our experimental system.

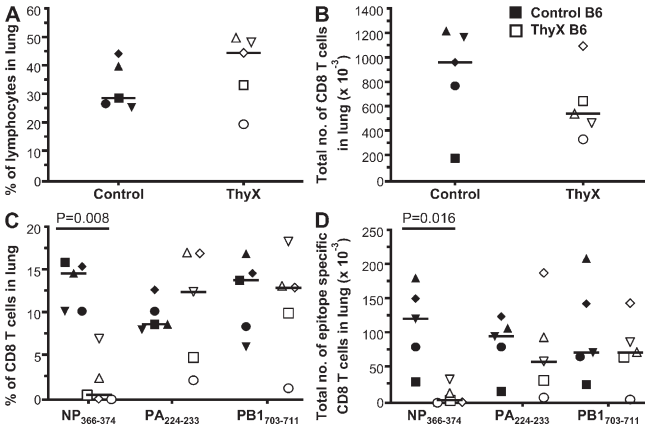

Figure 7. Thymectomy results in the perturbation of the NP-specific repertoire of CD8 T cells in young C57BL/6 mice. Young naive C57BL/6 mice were thymectomized, rested for 7 mo, and then intranasally infected with influenza virus. Lung tissue was harvested from both groups of mice 11 d after infection. The frequencies and absolute numbers of CD8 T cells detected in the lungs of individual infected mice are shown in A and B. The frequencies and absolute numbers of CD8 T cells positive for the influenza virus MHC class I tetramers NP ($NP_{366-374}/D^b$), PA ($PA_{224-233}/D^b$), or PB1 ($PB1_{703-711}/K^b$) detected in the lungs of individual infected mice are shown in C and D. Open and closed symbols represent individual thymectomized (ThyX) and control mice, respectively ($n = 5$ for each group). Bars indicate the medians calculated from the groups of control and thymectomized mice. Significance was assessed using the Mann-Whitney rank test (two-tailed, 95% confidence).

The development of protective heterosubtypic immunity in aged mice correlates with the development of a strong NP response after primary infection

To determine the impact of the age-associated loss in responsiveness to NP on protective immunity, we further exploited the well-developed mouse model to directly examine the response of individual young and aged mice infected de novo with the PR8 strain of influenza virus (H1N1) on their ability to clear virus after heterosubtypic challenge with the x31 strain (H3N2). This system of priming and challenge avoids the complication of cross-neutralizing antibodies and allows heterosubtypic cellular immunity to be examined directly. Thus, aged and young mice were intranasally infected with PR8 virus (300 EID_{50}), their capacity to respond to NP was measured by tetramer analysis of peripheral blood lymphocytes, and then they were allowed to recover. After 30 d, the mice were challenged intranasally with x31 virus (30,000 EID_{50}), and analyzed for NP and PA reactivity by tetramers and viral titers measured as a readout of the heterosubtypic response. As it is not possible to carry out kinetic studies of viral clearance on an individual mouse, we chose a single

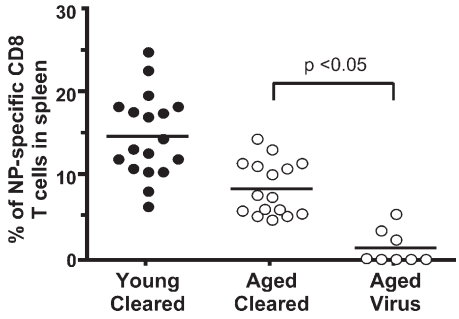

Figure 8. Protective heterologous immunity in aged mice correlates with the ability to develop a primary response to the NP epitope. Young and aged C57BL/6 mice were first intranasally infected with 300 EID_{50} PR8 (H1N1), and then were intranasally challenged with 30,000 EID_{50} x31 (H2N3) 30 d later. On day 7 after challenge, lung tissue was harvested from infected mice for measurement of viral titers, and splenic tissue was harvested for tetramer analyses, as described in the Materials and methods. Each symbol represents the frequency of splenic CD8 T cells specific for NP as determined for individual young (open circles) and aged (closed circles) mice that had either cleared or not cleared virus. The means of 18 young mice that cleared virus, 14 aged mice that cleared virus, and 7 aged mice that failed to clear virus are indicated.

time point for analysis when virus is typically cleared in young mice. The data shown in Fig. 8 confirmed that virus was cleared on day 7 in all young mice, but only a subset of aged mice. Importantly, there was a correlation between the ability of the aged mice to clear the challenge virus by day 7 and the development of an NP response after primary infection. Aged mice that had <5% NP-specific cells failed to clear virus, and aged mice with NP-specific cells between 5 and 15% had cleared virus on day 7. These data indicate the functional significance of the age-associated loss of the ability to respond to NP after de novo influenza virus infection.

DISCUSSION

We have used an influenza virus infection model to address the relationship between the naturally occurring age-associated decline in repertoire diversity and the response of aged animals to a newly encountered pathogen. We showed using in vivo limiting dilution analysis that the precursor frequency of T cells specific for NP in naive, young C57BL/6 mice is ~10-fold lower than the precursor frequency of T cells specific for PA. The CD8 T cell responses to NP and PA in young mice are equidominant (31, 48) and are mediated predominantly by a restricted repertoire of Vβ8.3$^+$ T cells and a diverse repertoire of Vβ7$^+$ T cells, respectively (36, 38). However, analysis of the response of aged mice to de novo influenza virus infection exhibited a markedly altered immunodominance, characterized by a preferential loss of NP reactivity. In addition, TCR repertoire analysis with both TCR Vβ antibodies and CDR3 spectratyping showed age-associated perturbations in the repertoire of NP- and PA-specific CD8 T cells that were unique in individual mice. Importantly, the impact of age-related contraction of the T cell repertoire on the CD8 T cell response to influenza virus infection was mimicked by thymectomy, which accelerated the age-associated loss of NP responsiveness. Finally, the age-dependent failure to develop an NP-specific response after primary infection generally correlated with poor heterosubtypic protection. Together, these data provide experimental evidence that the age-associated decline in CD8 T cell repertoire diversity can greatly impact the response to new infections, and the development of heterosubtypic immunity. Importantly, perturbations in the repertoire of T cells specific for influenza virus epitopes for which there is a low precursor frequency and limited TCR diversity, lead to the selective development of holes in the repertoire for a typically immunodominant viral epitope.

T cell repertoire is frequently characterized by either exhaustive sequence analysis of individual cells or spectratype analysis of bulk populations of T cells, and different information is obtained by the two techniques. The repertoires of Vβ8.3$^+$ NP-specific T cells and Vβ7$^+$ PA-specific T cells in C57BL/6 mice have been extensively characterized by clonal sequencing. 45 β-chain sequences have been reported for Vβ8.3$^+$ NP-specific T cells, 3 of which were public (shared between individuals), and 241 different β-chain sequences have been reported for Vβ7$^+$ PA-specific T cells, none of which were public. Individual mice expressed an average of 8 different NP Vβ8.3 sequences and 21 different PA Vβ7 sequences (36, 38, 39). More recently, sequence analysis of NP-specific T cells was extended to include those expressing Vβ4$^+$ TCRs, and only four different clonotypes were identified, leading to the conclusion that the Vβ4 component of the primary NP response in young mice was restricted and public, analogous to the Vβ8.3 component (37). A broader picture of repertoire diversity can be assessed by spectratype analysis, as CDR3 diversity reflects the overall complexity of T cell populations. Whereas naive repertoires show a characteristic Gaussian distribution of CDR3 length diversity, oligoclonal responses are reflected by the emergence of distinct peaks (46). These peaks sometimes reflect expansion of a single clone, but this is not the case for the responses to NP and PA. For example, for the dominant Vβ8.3 and Vβ7 responses to NP and PA in C57BL/6 mice, there have been shown to be several different sequences with the same CDR3 length represented within the dominant 9- and 6-aa spectratype peaks, respectively (36, 38). Thus, our spectratype analysis was comprehensive in that it spanned all TCR Vβ families. The response in individual young mice to both NP and PA was diverse. This heterogeneity in the fine specificity of epitope-specific T cells is predicted by the previous demonstration that the naive repertoire to specific peptides in individual mice is unique (48). Because of stochastic age-associated declines in the naive repertoire, the extreme heterogeneity in individual aged mice is not unexpected. Importantly, because of the low precursor frequency and the comparatively restricted diversity in the TCR repertoire of NP-specific T cells in young mice, approximately half of aged mice developed a hole in the repertoire and were unable to generate an effective response to NP.

T cell responses to de novo antigens are not dependent solely on naive precursors. Because T cell recognition is highly degenerate (49, 50), and there is a surprising degree of cross reactivity in the T cell responses to seemingly unrelated viral antigens (51, 52), it has been suggested that fortuitously cross-reactive memory cells may make an important contribution to the response to newly encountered antigens in mice as the naive repertoire becomes increasingly constrained with age (53, 54). Indeed, aged mice show a high proportion of memory–phenotype T cells in the periphery. This complicates the simple interpretation of our data, in that our analyses did not distinguish the relative contributions of naive and cross reactive memory cells to the antiviral CD8 T cell response in individual mice. This does not, however, lessen the significance of our data which demonstrate that age-associated loss of repertoire diversity dramatically impacts the response to de novo antigens, and can, in some cases, lead to loss of responsiveness to a particular epitope. Moreover, it is likely that, because of structural constraints, the NP epitope elicits only a restricted repertoire of naive T cells (39), and is less likely to induce cross reactivity in heterologous memory cells. Thus, cross reactive memory cells may only make a minor contribution to the NP response in naive aged mice. Defining the exact role of these cells in the response of

aged mice to primary influenza virus infection will require further study.

T cell clonal expansions frequently develop in aged individuals, and contribute to the age-associated decline in repertoire diversity (21, 28, 29, 55, 56) and to compromised antiviral CD8 T cell immunity (50, 56). Aged mice showing signs of clonal expansions indicated by Vβ perturbations in the peripheral blood CD8 T cells were excluded from our study. However, because the NP-specific precursor repertoire appears sensitive to any perturbation in the naive repertoire, it is likely that the presence of clonal expansions would further constrain the diversity of the antiviral repertoire as described in the current studies (21).

How do our data fit in with other studies on the impact of aging on the de novo response to influenza virus infection? Reduced primary CD8 T cell responses to influenza virus in aged mice, including delayed viral clearance, have been reported (12, 57, 58), consistent with generalized defects in the immune response to influenza virus in elderly people (9). In a previous study, it was shown that aged mice had a reduced frequency of NP-specific T cells and a corresponding reduction in overall NP-specific effector function, but responses to other epitopes were not examined (12). In contrast, other reports found no functional defect in the response of aged naive CD8 T cells (14, 15). Consistent with this, we have shown that despite being found in small numbers, NP-specific CD8 T cells elicited in infected aged mice are as functional as those elicited in younger mice, as determined by IFNγ ELISpot analysis (unpublished data). Our data reconcile this apparent inconsistency by showing that the loss of CD8 T cell responses to particular epitopes can be explained by age-associated development of "holes" in the naive T cell repertoire, rather than functional defects, which is consistent with the findings that the CD8 cells present in aged mice are fully functional.

What are the consequences of a loss of reactivity to NP and corresponding shifts in immunodominance for protective immunity? In the current studies, we examined the impact of the loss of NP responsiveness on the immediate recall response to secondary influenza virus infection and showed a general correlation between the ability to clear heterosubtypic virus and the generation of a strong NP response after primary infection. This was surprising to us, as we anticipated that in the absence of the immunodominant NP epitope, compensatory responses to known or hidden epitopes would emerge (42, 59). However it has been shown that epitopes may vary in protective efficacy (60).

In terms of repertoire considerations, in our studies, aged mice generated a comparable CD8 T cell response in terms of numbers of CD8 T cells elicited in the lung and BAL after infection. However, there was a clear shift in immunodominance. Because we only measured responses to three influenza virus epitopes (NP, PA, and PB1), in many cases we failed to identify the viral epitopes to which the aged mice responded. Despite the declining responsiveness to NP, aged mice appeared able to control the infection. However, consistent with other reports (12, 57, 58), viral clearance was delayed in aged mice (unpublished data), likely a consequence of shifts in immunodominance. Shifts in immunodominance can also have unexpected effects on the response to other epitopes. For example, it has been suggested that in the face of an enhanced PA response, the NP response is suppressed (40). However, this was not the case in the current studies, as the NP response remained either greatly diminished or absent in a high percentage of aged mice after infection with a virus from which the PA/D^b epitope could not be generated (41, 42). Whereas it has been shown that the primary responses to NP and PA are relatively equidominant in C57BL/6 mice, the NP response is extremely immunodominant in the recall response (47, 61–63). Thus, there may be an important impact of age-associated loss of NP responsiveness on the secondary recall responses of those mice with PA-dominated responses after primary infection. For example, we have previously shown that vaccination of young mice with the PA peptide elicited a strong epitope-specific response, but failed to protect upon challenge with wild-type virus. In addition, it appeared that vaccination with PA actually exerted a detrimental effect on subsequent protection under certain circumstances (40, 63). Therefore, the dominant primary response to PA in aged mice may have a profound impact on protective immunity.

However, because the impact of aging on the development of long-lasting protective immunity is complex, there are likely other contributing factors in addition to effects on the CD8 T cell repertoire. For example, CD4 signals are essential during the priming phase for the generation of good memory (64–66). As CD4 T cell function is impaired in aged mice (5, 67–71), it is essential to determine how this contributes to impaired development of protective immunity after de novo infection of aged mice. We are currently examining this question.

Collectively, our findings may have important implications for the future design of cellular vaccines intended for the elderly human population. In addition to declining innate immunity and impaired humoral immune responses, the loss of T cell repertoire diversity needs to be considered. The potential for the development of holes in the T cell repertoire of aged individuals argues against the feasibility of epitope-based vaccination strategies for the elderly. In addition, the emphasis on improving adjuvants to overcome the defective immunity of the aged may be inappropriate, as even the most potent adjuvants will not augment the normal protective CD8 T cell response to infection in the absence of antigen-specific precursors, and may even result in priming of non-protective or detrimental responses in aged individuals. Rather, newer strategies need to focus on boosting preexisting memory T cell responses present within aged individuals. In addition, it may be desirable to prime cellular immunity before severe loss of thymic output, suggesting that more vaccinations during middle age may be indicated. Therapeutic approaches for improving survival and maintenance of naive T cells, prolonging thymic output, and reconstituting the

repertoire of the elderly through hematopoietic stem cell reconstitution should also be considered (6, 8, 22, 72–74).

MATERIALS AND METHODS

Viruses, mice, and infections. Influenza viruses A/HK-x31 (x31, H3N2), A/PR8/34 (PR8, H1N1), and a PA-deficient influenza virus (42) were grown, stored, and titrated as previously described (75). C57BL/6J (CD45.2$^+$), B6.SJL-*Ptprc*^{a} Pep3/BoyJ (CD45.1$^+$), and B6.129P2*Tcrb*^{tm1Mom}*Tcrd*^{tm1Mom} (TCRβ/δ$^{-/-}$, CD45.2$^+$) mice were obtained from the Trudeau Institute animal facility or purchased from The Jackson Laboratory and maintained under specific pathogen–free conditions. Peripheral blood lymphocytes of all aged mice were prescreened for T cell clonal expansions, and those that exhibited TCR Vβ8 staining ± 3 SD over that observed with young C57BL/6 mice were omitted from the study. All animal procedures were approved by the Institutional Animal Care and Use Committee at Trudeau Institute. Thymi were removed from 4–5-wk-old C57BL/6 mice under nembutal anesthesia (Abbott) and mice were rested for ≥7 mo before use. Sex-matched young (6–12-wk-old) and aged (>18-mo-old) mice were anesthetized with 2,2,2-tribromoethanol and infected intranasally with 300 EID_{50} of either x31 or PR8 influenza virus, or 600 EID_{50} of the PA-deficient influenza virus. For heterologous protection studies, young and aged C57BL/6 mice were first intranasally infected with 300 EID_{50} PR8. Mice were bled at 10 d after infection to assess individual responsiveness to the NP epitope via tetramer staining and then intranasally challenged with 30,000 EID_{50} x31 at 30 d after infection. 7 d after challenge, viral titers were determined using an influenza infective foci assay, and spleen cells were stained to determine the percentage of NP-, PA-, and PB1-specific cells using tetramers.

MHC tetramer reagents and analysis. MHC class I peptide tetramers specific for influenza virus $NP_{366–374}/D^b$, $PA_{224–233}/D^b$, and $PB1_{703-711}/K^b$ were generated by the Molecular Biology Core Facility at the Trudeau Institute, as previously described (76). Tetramer staining was performed for 1 h at room temperature, followed by incubation with anti–CD8-PerCP, and data were collected on a Becton Dickinson FACSCalibur flow cytometer. Data were analyzed using FlowJo (Tree Star, Inc.) software.

TCR Vβ chain usage analysis. TCR Vβ chain usage by viral-specific CD8 T cells was determined by using a panel of FITC- and PE-conjugated anti-TCR Vβ antibodies that included Vβ2, Vβ4, Vβ5.1/5.2, Vβ7, Vβ8.3, and Vβ13 (all antibodies were purchased from BD Biosciences).

Cell sorting. For the isolation of influenza-specific CD8 T cells from individual young and aged infected mice, cells were stained with APC-conjugated $NP_{366–374}/D^b$ tetramer, PE-conjugated $PA_{224–233}/D^b$ tetramer, anti-CD8-FITC, and PE-Cy5-conjugated anti-CD19 and anti-CD4 ("dump" channel). Samples were then sorted on a FACSVantage flow cytometer with DiVa options (BD Biosciences) into CD8/NP tetramer$^+$ and CD8/PA tetramer$^+$ populations. The purity after sorting was ≥95%.

Spectratype analysis. Repertoire analysis was performed by assessing CDR3 length distribution, using 5′ Vβ and 3′ Cβ primers and a modification of the protocol described by Pannetier et al. (46, 76). Total RNA was extracted from sorted cells using the RNeasy Mini kit (QIAGEN) and eluted in a volume of 30 μl of DEPC-treated water. Using the RETROscript kit (Ambion), cDNA was synthesized from the RNA, in a 40-μl reaction following the manufacturer's instructions. Primer sequences for mouse Vβ and Cβ segments were synthesized at Integrated DNA Technology, Inc. 1 μl of cDNA was added to a final 50-μl mixture containing 5 μl of GeneAmp 10X PCR Buffer II (Applied Biosystems), 0.2 mM of dNTP mix (Invitrogen), 1.5 mM of MgCl2 (Applied Biosystems), 10 pmol of 5′ Vβ primer (77), 10 pmol of 3′ Cβ primer (CTTGGGTGGAGTCACATTTCT), and 1.25 U of AmpliTAQ Gold DNA polymerase (Applied Biosystems). PCR was run as follows: 10 min at 95°C, 35 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 7 min. 2 μl of the PCR products were then used as template for an elongation reaction (run-off reaction) using only a 6-FAM–labeled 3′ Cβ primer in a 50-μl reaction. PCR was performed as follows: 10 min at 95°C, 10 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 7 min. PCR products (1 μl) from run-off reactions were mixed with loading buffer containing GeneScan 500 ROX size standard (Applied Biosystems) and denatured at 95°C for 2 min. Samples were then applied to an ABI Prism 310 Genetic Analyzer, and GeneScan software (Applied Biosystems) was used to analyze the data.

Adoptive transfer of CD8 T cells. For the studies described in Figs. 1 and 2, CD8 T cells were enriched from the spleens of young naive C57BL/6 mice (CD45.2$^+$) via negative selection columns (R&D Systems). Purified CD8 T cells were pooled and transferred, along with CD8-depleted splenocytes from young congenic naive B6.SJL mice (CD45.1$^+$), into young TCR-deficient hosts (TCRβδ$^{-/-}$) via intravenous injection. Recipient mice were infected with influenza virus 1 d after transfer, and BAL and lung tissue were harvested for analyses at various days after infection. Day 14 was determined to be the peak of the response in adoptive hosts (unpublished data). Responding donor CD8 T cells were identified and enumerated through the use of antibody staining and MHC class I tetramers.

Data analyses. Statistical significance was calculated using the nonparametric Mann-Whitney rank test using Prism 4 software (GraphPad Software). P values <0.05 were considered significant.

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