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Clonal Expansions and Loss of Receptor Diversity in the Naive CD8 T Cell Repertoire of Aged Mice[1]

Mushtaq Ahmed,[2] Kathleen G. Lanzer, Eric J. Yager, Pamela S. Adams, Lawrence L. Johnson, and Marcia A. Blackman[3]

There are well-characterized age-related changes in the peripheral repertoire of CD8 T cells characterized by reductions in the ratio of naive:memory T cells and the development of large clonal expansions in the memory pool. In addition, the TCR repertoire of naive T cells is reduced with aging. Because a diverse repertoire of naive T cells is essential for a vigorous response to new infections and vaccinations, there is much interest in understanding the mechanisms responsible for declining repertoire diversity. It has been proposed that one reason for declining repertoire diversity in the naive T cell pool is an increasing dependence on homeostatic proliferation in the absence of new thymic emigrants for maintenance of the naive peripheral pool. In this study, we have analyzed the naive CD8 T cell repertoire in young and aged mice by DNA spectratype and sequence analysis. Our data show that naive T cells from aged mice have perturbed spectratype profiles compared with the normally Gaussian spectratype profiles characteristic of naive CD8 T cells from young mice. In addition, DNA sequence analysis formally demonstrated a loss of diversity associated with skewed spectratype profiles. Unexpectedly, we found multiple repeats of the same sequence in naive T cells from aged but not young mice, consistent with clonal expansions previously described only in the memory T cell pool. Clonal expansions among naive T cells suggests dysregulation in the normal homeostatic proliferative mechanisms that operate in young mice to maintain diversity in the naive T cell repertoire. *The Journal of Immunology,* **2009, 182: 784–792.**

Immune function declines with aging, characterized by an increased susceptibility to new infections and reduced responsiveness to vaccination (1–6). Because a diverse repertoire of naive peripheral T lymphocytes is essential for efficient generation of immune responses to new infections and vaccines, it has been proposed that age-associated reductions in naive repertoire diversity contribute to the characteristic decline in immune function in the elderly (7–11).

Naive and memory T cells have been shown to occupy separate niches in the periphery and the ratio of naive and memory peripheral T cells is relatively stable through adulthood. Furthermore, the separate niches are maintained by independent mechanisms. Whereas the memory pool is influenced by Ag experience, maintenance of the naive T cell pool is independent of foreign Ag and is controlled by thymic export and peripheral homeostasis (12–15). However, both in mouse and humans, the peripheral repertoire becomes dramatically skewed with age in favor of memory T cells (16, 17). This is due to both thymic and peripheral events. Thymic involution as a consequence of aging results in decreased thymic output necessary to replenish the peripheral naive T cell pool (3, 18–22). In addition, peripheral events, including the accumulating Ag experience of the host and the development of clonally expanded populations of CD8 T cells are thought to contribute to the relative increase in proportions of memory compared with naive T cells (23–27). Thus, the overall peripheral repertoire becomes progressively biased toward memory cells as fewer new T cells are produced and naive T cells continue to be recruited into the memory pool as a consequence of Ag exposure.

Despite these pressures, naive T cells persist with age and maintenance of the naive pool in the face of decreasing thymic export becomes increasingly dependent on homeostatic proliferation (16, 28–30). Although T cell maturation takes place predominantly in the thymus, thymic emigrants undergo further phenotypic and functional maturation in the periphery after low-affinity interactions with self- peptide-MHC complexes (31–37). Consistent with this, naive T cells have been shown to proliferate at a low level, although significantly less than the memory pool (38, 39). Whereas numbers of peripheral naive T cells can be maintained by homeostatic proliferation, it has been suggested that peripheral homeostatic proliferation of naive T cells in the face of declining thymic export will result in loss of repertoire diversity in aged mice (18).

T cell specificity is determined by the TCR. The diversity of the TCR repertoire is generated in the thymus through imprecise assembly of V, D, J, and C gene segments to form the α- and β-chains of the TCR (40). A major contribution to repertoire diversity is the third hypervariable region of the receptor chains, also referred to as the third CDR (CDR3). As a consequence of the imprecise joins between the V, D, and J regions, there is a range of CDR3 sizes in a population of T cells that vary in length by 10 or more amino acids (41). This diversity can be analyzed by TCR spectratyping, which measures the sizes of the CDR3s in a pool of cells by analyzing Vβ-Cβ RT-PCR products that have been labeled with a runoff reaction on a sequencing gel (10, 42–44). Typically, a histogram of CDR3 lengths from a diverse population of T cells is symmetrical and bell-shaped, generally referred to as Gaussian. Deviations from a Gaussian distribution are often seen

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Table I. *Oligonucleotide sequences of spectratyping primers*

Vβ and Cβ Primers	Sequence 5′–3′	Size of Primer (bp)	Distance to CDR3[a] (bp)
Vβ1	CT GAA TGC CCA GAC AGC TCC AAG C	24	83
Vβ5.1	CAT TAT GAT AAA ATG GAG AGA GAT	23	135
Vβ8.1	CAT TAC TCA TAT GTC GCT GAC	21	141
Vβ8.3	T GCT GGC AAC CTT CGA ATA GGA	22	127
Vβ9	TCT CTC TAC ATT GGC TCT GCA GGC	24	57
Vβ10	ATC AAG TCT GTA GAG CCG GAG GA	23	48
Vβ11	G CAC TCA ACT CTG AAG ATC CAG AGC	25	64
Vβ12	G ATG GTG GGG CTT TCA AGG ATC	22	117
Vβ16	C ACT CTG AAA ATC CAA CCC AC	21	58
Cβ	CTT GGG TGG AGT CAC ATT TCT	22	60

[a] The length in bp for V primers is counted from the first base in the V gene corresponding to the 5′ end of the primer through to the last base of the V gene before the CDR3 region. The length in bp for the C primer is counted from the first base of the J gene after the CDR3 region through to the last base in the C gene corresponding to the 5′ end of the primer (42).

within $CD44^{high}$ (memory) T cells, a consequence of expansion of a particular component of the population, e.g., as a result of Ag exposure or age-associated clonal expansions. In young individuals, the naive repertoire remains diverse and Gaussian. However, perturbed spectratype profiles have been demonstrated among naive CD4 T cells of aged humans (29), and estimates of receptor diversity among naive CD4 T cells showed a precipitous decline after 70 years of age (16). These data are consistent with the hypothesis that homeostatic proliferation in the absence of new thymic emigrants results in constraints in naive T cell repertoire diversity (18).

In this study, we have characterized repertoire diversity of naive CD8 T cells from young and aged mice. Spectratype profiles of naive CD8 T cells from individual young mice showed a Gaussian distribution, indicative of a diverse repertoire, as expected. In stark contrast, there were extensive perturbations in the spectratype profiles of naive CD8 T cells from individual aged mice, suggestive of a skewed repertoire. We conducted sequence analysis of individual clones from selected peaks within the Vβ8.3-Cβ spectratypes of young and aged mice to directly assess repertoire diversity. Unexpectedly, sequence analysis revealed evidence for clonal expansions in the naive CD8 T cell pool in aged mice. These data provide the first report of age-associated clonal expansions in naive T cells and support the idea that in the absence of new thymic emigrants homeostatic proliferation results in unequal maintenance of individual clones and loss of repertoire diversity.

Materials and Methods

Mice

Aged female C57BL/6J mice (17 mo old) were purchased from the National Institute of Aging and used at 19 mo of age. Young female C57BL/6 mice were purchased from The Jackson Laboratory or bred at the Trudeau Institute and used at 6 mo of age. Animals were housed under specific pathogen-free conditions. All animal procedures were performed in compliance with the Institutional Animal Care and Use Committee at the Trudeau Institute.

Cell enrichment and sorting

Single-cell suspensions of splenocytes were lysed of erythrocytes and B cells were removed by direct panning with 100 μg/ml goat anti-mouse IgG. The remaining cells were stained with fluorochrome-conjugated mAbs specific for CD19, CD4, CD8α, and CD44 in the presence of Fc block. Samples were then sorted on a FACSVantage flow cytometer with DIVA options (BD Biosciences) into $CD8^+CD44^{low}$ (naive) and $CD8^+CD44^{high}$ (memory) populations. mAbs were purchased from either BD Biosciences or eBioscience.

RNA extraction and cDNA synthesis

Total RNA was extracted from sorted cells (0.78- 1.5 × 10^6 cells from young mice and 0.95–4.0 × 10^5 cells from aged mice) using the RNeasy Mini kit (Qiagen) and eluted in a volume of 30 μl of diethyl pyrocarbonate-treated water. Using the RETROscript kit (Applied Biosystems/Ambion), cDNA was synthesized from equal quantities of total RNA (160 ng) in a 40-μl reaction following the manufacturer's instructions.

DNA spectratype analysis

Spectratyping analysis was conducted with modifications of the protocol described by Pannetier et al. (42). Primer sequences for mouse Vβ and Cβ segments were synthesized at Integrated DNA Technologies. One microliter of cDNA was added to a final 50-μl mixture containing 5 μl of GeneAmp 10× PCR Buffer II (Applied Biosystems), 0.2 mM dNTP mix (Invitrogen), 1.5 mM $MgCl_2$ (Applied Biosystems), 10 pmol of 5′ Vβ and Cβ primers (Ref. 42; Table I), 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. Two microliters of the PCR products were then used as template for an elongation reaction (runoff reaction) using only a 6-FAM-labeled 3′ Cβ primer in a 50-μl reaction. PCR was conducted as follows: 10 min at 95°C, 10 cycles of 94°C for 45 s, 62°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 runoff 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 Applied Biosystems Prism 310 Genetic Analyzer (Applied Biosystems). GeneScan software version 3.1 (Applied Biosystems) was used to analyze the spectratype data.

Repertoire analysis by isolation of TCR Vβ PCR products and CDR3 junctional sequencing

The TCR Vβ PCR products corresponding to selected Vβ8.3 spectratype profiles from one young and three aged mice were resolved on a 6% denaturing acrylamide gel. The PCR products were silver-stained using a Silver Sequence DNA Staining Kit (Promega). DNA bands of interest were excised and purified using a QIAEX II kit (Qiagen). One microliter of eluted PCR products was reamplified using the SuperTaq Plus Polymerase PCR kit (Applied Biosystems/Ambion). PCR was run as follows: 2 min at 94°C, 20 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. These PCR products were further resolved on a 15% nondenaturing acrylamide gel and subjected to DNA silver staining. The bands of interest were excised, purified, and reamplified by PCR, as described above. The amplification products were purified using a PureLink Gel Extraction Kit (Invitrogen). The purified PCR products were cloned into vector pCR2.1-TOPO using a TOPO TA cloning kit (Invitrogen). Ligated products were transformed into One-Shot OmniMAX 2T1 Phage-Resistant Cells (Invitrogen), according to the manufacturer's instructions, to achieve maximum transformation efficiency. By this approach, a spectrum of clones representative of all band species within a spectraype peak was generated. DNA sequencing of the clones was performed at Genewiz using plasmid DNA or directly on $LacZ^-$ colonies. TCR CDR3 sequences derived from each peak were aligned and analyzed using the CLUSTALW2 program and multiple alignment tool at http://www.ebi.ac.uk/Tools/clustalw2/index.html.

Statistical analysis

Comparative analysis of spectratype profiles from young and aged mice. The normalized value of peak intensities for individual aged mice was compared peak by peak with the normalized mean ± 3 SD for young mice.

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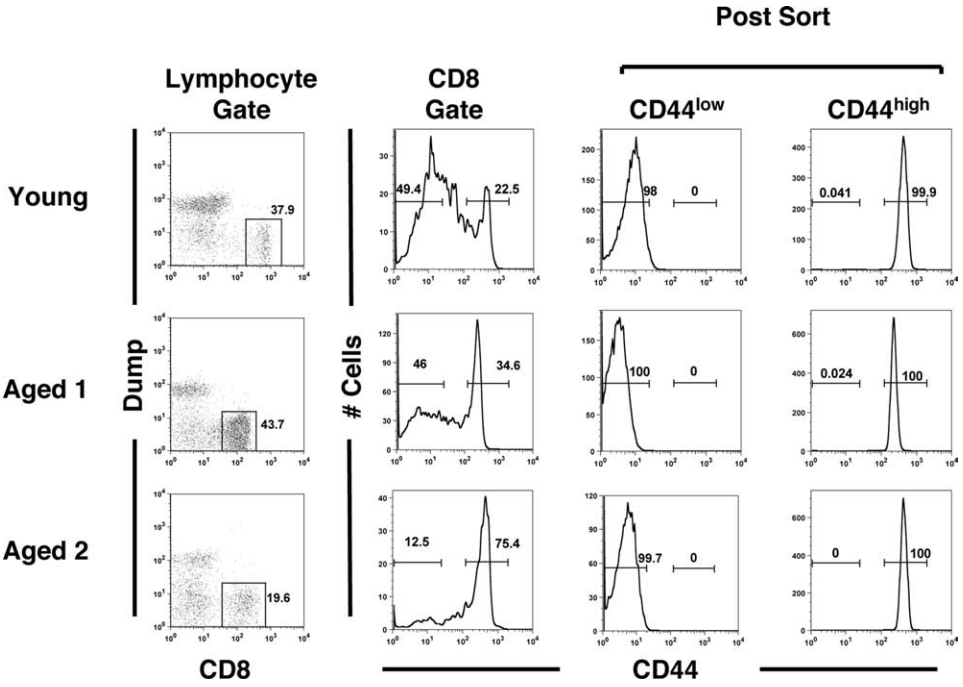

FIGURE 1. Highly purified populations of naive CD8 T cells can be sorted from individual aged mice. Gated CD8 T cells from a representative young and two individual aged mice were analyzed for CD44 expression. The data show that the proportion of naive ($CD44^{low}$) relative to memory ($CD44^{high}$) CD8 T cells varied in individual aged mice, but was consistently lower than in young mice. Despite the underrepresentation of naive CD8 T cells in aged mice, highly purified populations for repertoire analysis could be obtained by flow cytometric sorting.

The probability that a given peak within a spectratype would fall more than 3 SDs from the mean of the corresponding peak in the reference set is 0.01 under the null hypothesis that the spectratype was a random sample from the reference population. Under that hypothesis, the probability of finding at least one "skewed" peak within a given spectratype is $1 -$ (probability of 0 skewed peaks) $= 1 - 0.99^{(\text{no. of peaks within that spectratype})}$. Thus, the probability of an eight-peak spectratype drawn at random from the reference population being scored as skewed was calculated to be $1 - 0.99^8 = 0.077$. The number of skewed spectra for each Vβ was noted for each aged mouse. Given the above probability (0.077) that a given spectratype of eight peaks would be scored as skewed under the null hypothesis, we next determined the binomial probability of finding one or more skewed spectratypes within the set of all Vβ spectratypes. This probability is $1 -$ (p of 0 skewed spectratypes) $= 1 - (1 - 0.077)^6 = 0.381$. From this, we calculated the probability that any mouse would have three or more skewed spectratypes simply by chance to be <0.01.

Analysis of sequence diversity. Based on the concepts outlined by Jost (45), we chose the Shannon diversity index as an appropriate measure of diversity within the sequence sets. We then followed the procedure outlined in the study by Hutcheson (46) to compare diversity indices for different sequence sets statistically by t test. Thus, a set of $n = 50$ total sequences was classified as n_1 of type 1, n_2 of type 2, …., n_s of type s, yielding s different sequences among the $\sum n_i = N$ total sequences. The diversity index H for each aged and young sequence set was calculated by the well known formula: $H = -\Sigma(n_i/N)\ln(n_i/N)$.

The variance of H (var H) was estimated from a series expansion as shown in the study by Hutcheson (46). We compared two diversity indices by t test using the df calculated by $df = (\text{var } H_1 + \text{var } H_2)^2/(\text{var } H_1^2/N_1 + \text{var } H_2^2/N_2)$ and $t_o = (H_1 - H_2)/(\text{var } H_1 + \text{var } H_2)^{1/2}$. We then compared t_o with tabulated t_α for the calculated df to determine the significance level.

Results

Highly purified naive CD8 T cells can be isolated from aged mice

The ratio of naive to memory T cells in the periphery declines with increasing age (16, 17, 47). Indeed, using CD44 as a cell surface marker to distinguish naive ($CD44^{low}$) and activated/memory ($CD44^{high}$) CD8 T cells (48), we found that the proportion of naive CD8 T cells was greatly diminished in the periphery of aged (19-mo-old) compared with young (6-mo-old) mice (Fig. 1). Despite the limited numbers of naive CD8 T cells in aged mice, we were nevertheless able to obtain substantial numbers ($>1 \times 10^5$) of highly purified naive CD8 T cells from individual aged mice using flow cytometric sorting (Fig. 1).

Spectratype profiles of naive T cells from aged mice are skewed

We next analyzed the diversity of the TCR repertoire in naive CD8 T cells from young and aged mice by Vβ spectratype analysis. To compare individual mice, RNA was prepared from similar numbers of FACS-sorted $CD44^{low}$ (naive) CD8 T cells from each mouse, and cDNA was synthesized from 160 ng of RNA from each mouse. Analysis of β-actin amplicons confirmed the quality and quantity of the cDNA from individual mice (data not shown).

The cDNA samples were subjected to spectratype analysis using 5′ Vβ primers for nine different Vβ families and a 3′ Cβ primer (Table I). Spectratype analysis of naive CD8 T cells from five individual young mice and nine individual aged mice is shown in Fig. 2. As expected for the young mice, for each Vβ family, a Gaussian pattern with an average of six to eight peaks, spaced by three nucleotides, was observed. However, the spectratype profiles for the aged samples showed perturbations for many Vβs, which varied within individual aged mice.

To quantitate the degree of perturbation among the aged spectratype profiles, peak areas in each Vβ spectratype were compared against the distribution of a reference standard that was determined by averaging values obtained from the naive CD8 T cell component of the five young mice. The intensities of the peaks within each Vβ spectratype for each young and aged mouse were normalized to a value of 1. The mean and SD of each (normalized) peak for each Vβ was calculated for the set of young controls and the mean $\pm$ 3 SDs was plotted for each peak (shown by bars in Fig. 3). For each aged mouse (individual symbols in Fig. 3), the spectratype for a given Vβ was compared peak by peak with the corresponding spectratype of averaged values for the young mice. A Vβ spectratype was scored as skewed for an aged mouse if one or more peaks within that spectratype fell outside 3 SDs of the corresponding young mouse reference peak. The data in Table II show that there was substantial skewing in all of the Vβs except Vβ16 and that the patterns of skewing varied for individual aged mice. The lack of skewing in Vβ16 may be a consequence of the large SD of the reference profiles for young mice seen uniquely for this Vβ (see Fig. 3). Among individual aged mice, there was a range of skewing among the Vβ families from less (skewing in four of nine Vβs, observed in aged mouse 3) to more (skewing in

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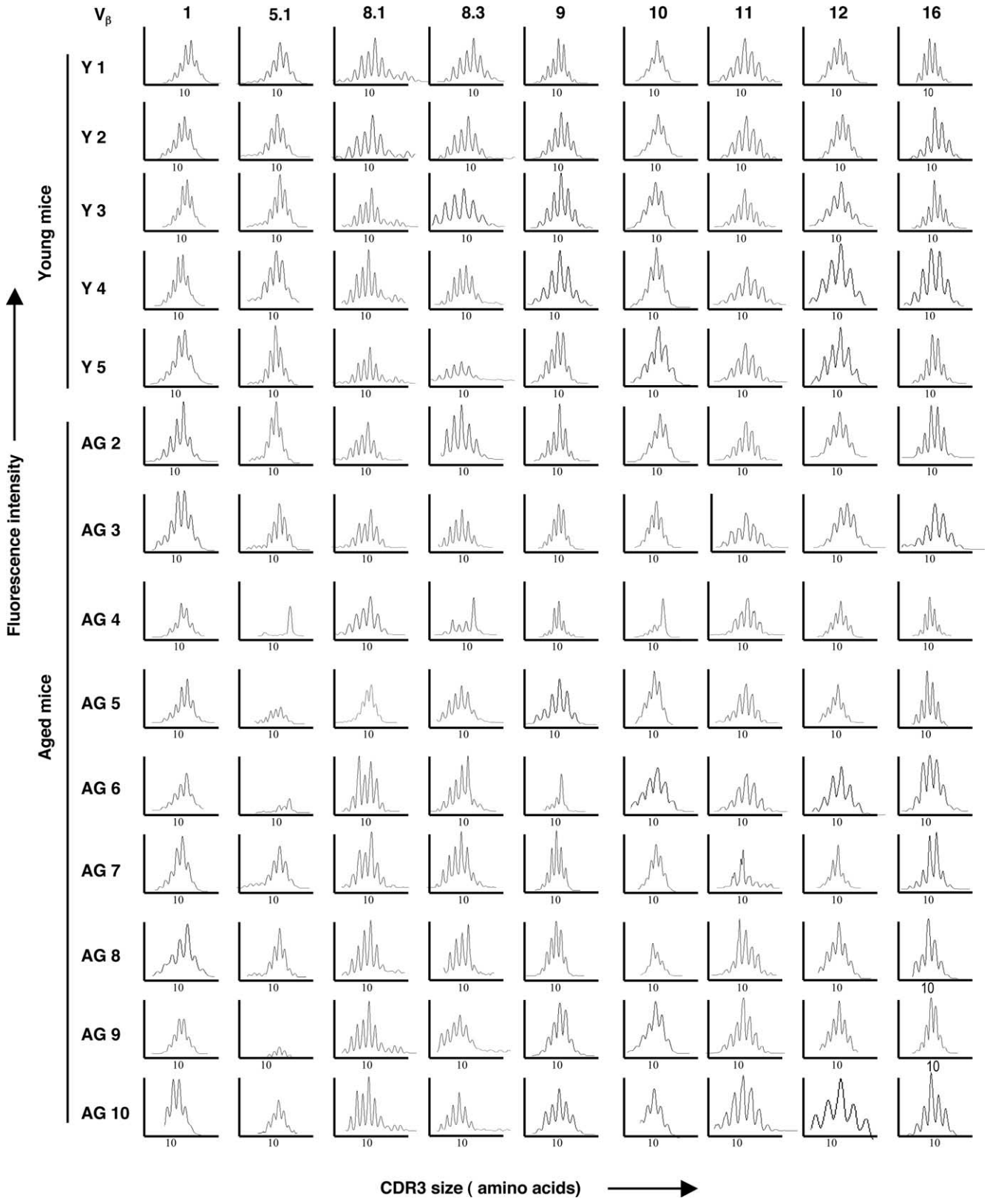

FIGURE 2. Spectratype analysis of naive CD8 T cells from aged mice shows distortions in the Gaussian distribution characteristic of young mice. Spectratype analysis for selected Vβ gene families for five young (Y1 through Y5) and nine aged (AG 2 through AG 10) mice is presented. CDR3 size is shown on the *x*-axis and relative peak area is shown on the *y*-axis. The peak corresponding to a CDR3 length of 10 aa is shown for each Vβ family.

eight of nine Vβs, observed in aged mice 5, 6, and 10). Thus, for each aged mouse, we rejected the null hypothesis that the presence of skewed spectratypes was random. All aged mice showed evidence of significant skewing of Vβ spectratypes (Table II).

The relationship between naive and memory CD8 T cell spectratype profiles for the same Vβ varied between individual mice and also varied within different Vβ spectratype profiles for the same mouse. The data demonstrated four distinct patterns (Fig. 4).

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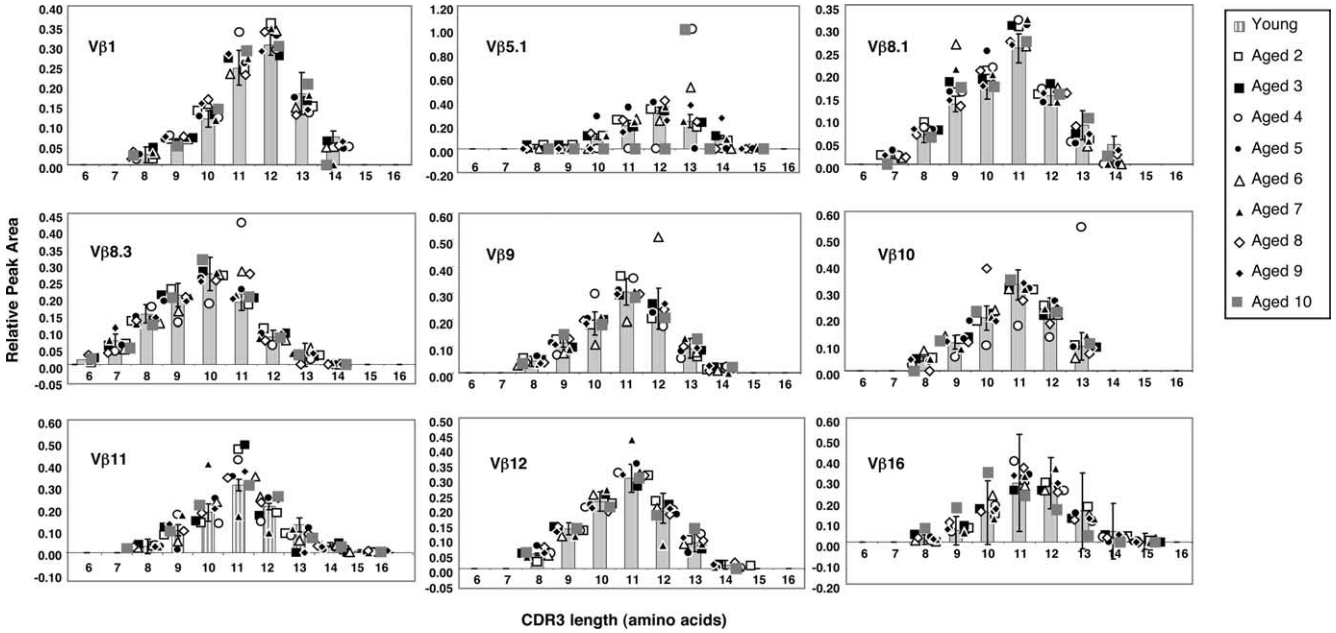

FIGURE 3. Skewing of the spectratype profiles for aged compared with young mice. The areas of individual peaks were normalized to a value of 1. The mean and SD of each normalized peak for each Vβ was calculated for the set of young controls and the mean ± 3 SD was plotted for each peak (shown by the bars). The normalized peak values for individual aged animals are plotted as distinct symbols.

In some cases, individual Vβ spectratype profiles were skewed for naive cells but not memory cells (Fig. 4*A*) and, in other cases, they were skewed for memory cells but not the corresponding naive cells (Fig. 4, *B*, *C*, and *F*). In another case, there was skewing in both naive and memory cells; the patterns were different (Fig. 4*I*). Finally, there was an example where there was a similar pattern of skewing in both the naive and memory cells (Fig. 4*E*). This latter pattern could not be attributed to contamination of the sorted population of naive cells with memory cells because this pattern was not consistent for all of the Vβ spectratype profiles from this mouse. For example, although the patterns for the naive and memory cells were similar for Vβ8.3 in aged mouse 4 (Fig. 4*E*), the patterns were very distinct for Vβ8.1 (Fig. 4*D*) and Vβ12 (Fig. 4*F*). This latter pattern in which skewing is similar for both the naive and memory CD8 T cell pool raises the possibility that, at least in some cases, clonal expansions in the memory pool may stem directly from perturbations in the naive pool. This possible mechanism merits further investigation. Importantly, however, this was only seen in one of nine cases examined. These results, taken together with published data (49–52), show that clonal expansions that are initiated in the naive pool do not appear to be a primary source of age-associated clonal expansions in the memory pool.

Sequence analysis of individual clones from selected peaks within the Vβ8.3 spectratype for naive cells from young and aged mice

Since a Gaussian spectratype profile in naive T cells is indicative of a diverse repertoire, we considered the possibility that the perturbations observed in naive T cells from aged mice were suggestive of reduced diversity. To formally link a skewed spectratype pattern with reduced diversity, we undertook sequence analysis of the specificities within an isolated band on a gel representing a spectratype peak of a given CDR3 size, as conducted previously, to determine the diversity of the naive repertoire in young mice (53). Each band in a spectratype peak, while uniform in size, is heterogeneous with respect to the nucleotide sequence composition (53, 54). Therefore, to determine whether the skewed profiles of aged mice are indicative of reduced repertoire diversity, we cloned and sequenced DNA isolated from selected single peaks from spectratype profiles of young and aged mice to estimate repertoire diversity.

We selected profiles generated from Vβ8.3-Cβ spectratyping from one young and three aged mice for further analysis (Fig. 5). As expected, the spectratype profile for the young mouse was not skewed, whereas the Vβ8.3 spectratype profiles for the aged mice showed variable skewing. For example, as indicated in Table II,

Table II. *Skewed Vβ spectratypes in aged compared with young mice*

	Vβ1	Vβ5.1	Vβ8.1	Vβ8.3	Vβ9	Vβ10	Vβ11	Vβ12	Vβ16	No. of Skewed Profiles[a]	p of Skewness[b]
A2	2[c]	2	5	1	1	0	6	1	0	7/9	<0.0001
A3	2	0	7	0	1	0	6	0	0	4/9	<0.0001
A4	4	6	6	6	3	6	7	0	0	7/9	<0.0001
A5	1	6	7	1	1	1	6	3	0	8/9	<0.0001
A6	3	6	4	4	4	1	6	2	0	8/9	<0.0001
A7	3	1	5	3	3	0	5	5	0	7/9	<0.0001
A8	5	5	2	2	0	4	3	2	0	7/9	<0.0001
A9	2	6	1	1	0	0	5	1	0	6/9	<0.0001
A10	3	6	3	1	1	1	3	2	0	8/9	<0.0001

[a] Number of skewed Vβ-Cβ spectratype profiles.
[b] Probability under the null hypothesis (refer to *Materials and Methods*) that Vβ spectratypes do not differ from those of young adults.
[c] Number of skewed peaks within each Vβ-Cβ spectratype profile.

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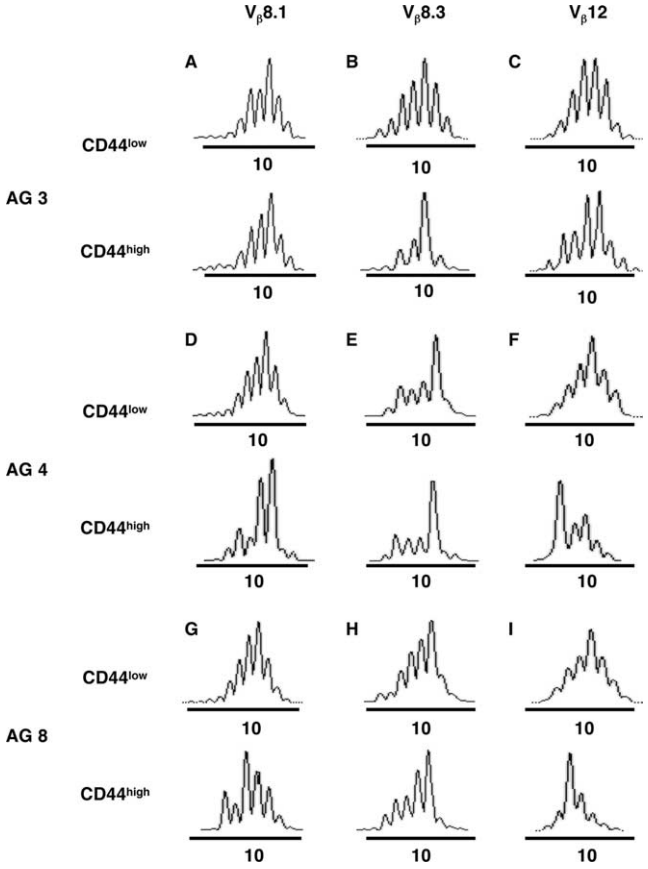

FIGURE 4. Lack of similarity between Vβ-specific spectratype profiles in naive and memory cells from individual aged mice. Spectratype analysis was conducted for FACS-sorted naive ($CD44^{low}$) and memory ($CD44^{high}$) CD8 T cells for selected Vβ gene families (Vβ8.1, Vβ8.3, and Vβ12) for three aged mice (AG 3, AG 4, and AG 8). CDR3 size is shown on the *x*-axis and relative peak area is shown on the *y*-axis. The peak corresponding to a CDR3 length of 10 aa is shown for each spectratype.

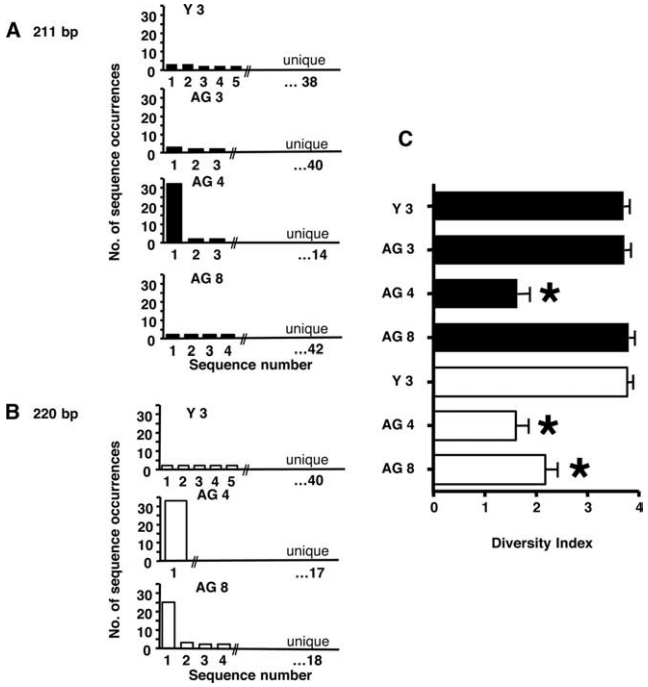

FIGURE 6. Overrepresentation of individual sequences reduces the diversity of TCR sequence usage among Vβ8.3$^+$ naive CD8 T cells in aged mice. DNA extracted from individual spectratype peaks (211 bp, ■, or 220 bp, □) of the Vβ8.3 spectratype profiles of one young and three aged mice, isolated as shown in Fig. 4, was cloned and sequenced. The data show the number of occurrences of individual base sequences (of ~50 total sequences) from the 211-bp peak (*A*) or 220-bp peak (*B*) isolated from spectratypes derived from young (Y) or aged (AG) mice. The numbers along the *x*-axis represent different DNA sequences and the number of times the same sequence occurs is plotted on the *y*-axis. Sequences that occurred only once are presented to the *right* of the hatches on the *x*-axis and are designated unique. *C*, The sequence frequency data, as shown in histograms in *A* and *B*, were used to calculate diversity indices with their SDs, as described in *Materials and Methods*. Bars from aged mice marked with an ∗ indicate diversity indices significantly reduced ($p < 0.001$) compared with those of young mice with the same bp length.

aged mouse 3 (AG 3) did not have a skewed spectratype profile for Vβ8.3. In contrast, the overall Vβ8.3 spectratype profile for aged mouse 4 (AG 4) was highly skewed (six of nine peaks skewed) and was moderately skewed for aged mouse 8 (AG 8) (two of nine peaks skewed). The DNA from the 211- and 220-bp peaks of individual mice was cloned and between 47 and 100 individual clones were sequenced. Analysis of diversity was based on the first (approximately) 50 sequences examined in each peak.

The sequence data are summarized in the histograms in *A* and *B* of Fig. 6, showing the number of occurrences (*y*-axis) of distinct sequences assigned identification numbers 1, 2, etc. (*x*-axis). Sequences that are not repeated are designated "unique" on the histogram. Analysis of both the 211-bp peak (Fig. 6*A*) and 220-bp peak (Fig. 6*B*) from the young mouse (Y 3) revealed that the sequences were mostly unique, and only a minority of the sequences were repeated two to three times. In contrast, sometimes a single sequence from aged mice occurred as many as 25–35 times (e.g., 211-bp sequences numbered 1 from AG 4 and 220-bp sequences numbered 1 from AG 4 and AG 8.) These data indicated the presence of clonal expansions in the naive Vβ8.3$^+$ CD8 T cell population of aged mice.

To determine a quantitative measure of diversity among individual mice as members of a population, we used a data analysis previously developed in disparate fields such as information science and ecology. We calculated measures of sequence diversity (diversity index) among sets of base sequences from young and

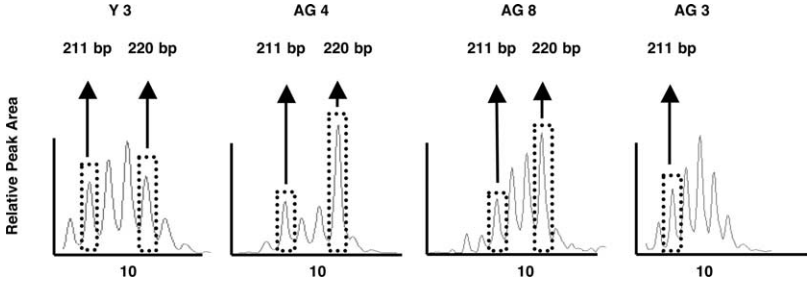

FIGURE 5. Isolation of individual Vβ8.3 spectratype peaks from young and aged mice. DNA from the 211- and the 220-bp spectratype peaks from one young (Y 3) and three aged (AG 4, AG 8, and AG 3) mice was separated by acrylamide gel electrophoresis and visualized by silver staining to allow isolation of DNA representative of a single CDR3 size before cloning and sequencing.

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aged mice and compared the indices statistically by *t* test. Thus, the sequence frequency data shown in *A* and *B* Fig. 6 were used to calculate diversity indices, as described in *Materials and Methods*, which are plotted (along with SD) in *C*. Bars marked with an asterisk indicate diversity indices significantly ($p < 0.001$) reduced compared with those of young control mice with the same bp length. The analysis shows that repertoire diversity in mice from which histograms are dominated by a single sequence occurring many times, indicative of clonal expansions, is significantly reduced compared with mice with histograms in which most sequences are unique.

Taking together the spectratype and sequence diversity analysis, the three aged mice examined fell into three patterns. One aged mouse (AG 3) showed minimal repertoire perturbation in terms of spectratype skewing (only four of nine Vβ spectratype profiles showed skewing and there were no skewed peaks in the Vβ8.3 spectratype profile, Figs. 3 and 5) and demonstrated comparable sequence diversity of the 211 Vβ8.3 peak to the young mouse (Fig. 6*C*). A second aged mouse (AG 4) was skewed in terms of spectratype profiles (seven of nine Vβ spectratype profiles showed skewing and there were six skewed peaks in the Vβ8.3 spectratype profile) and showed limited sequence diversity in both Vβ8.3 peaks that were analyzed (Fig. 6*C*). A third aged mouse (AG 8) was skewed in terms of spectratype profiles comparably to mouse AG 4 (seven of nine Vβ spectratype profiles showed skewing), although there were only two skewed peaks in the Vβ8.3 spectratype profile of AG 8. One peak (211 bp) demonstrated high sequence diversity comparable to that of the young mice and the second peak (220 bp) showed limited diversity (Fig. 6*C*). The overall conclusion is that skewing of the spectratype profile correlated with reduced repertoire diversity and evidence for clonal expansions. Thus, the overall tendency toward skewed spectratype profiles in aged mice (Figs. 2 and 3; Table II) reflects reduced repertoire diversity within the aged naive CD8 T cell pool (Fig. 6).

Discussion

We have formally evaluated the diversity of the naive CD8 T cell repertoire in aged compared with young mice. Spectratype profiles of naive T cells isolated from young mice typically show a Gaussian distribution indicative of a diverse repertoire. However, our analysis of naive CD8 T cells from aged mice shows extensive perturbations in the normally Gaussian Vβ spectratype profiles, suggestive of oligoclonal expansions and reduced repertoire diversity. Furthermore, DNA sequence analysis of clones obtained from isolated spectratype bands has revealed the presence of clonally expanded populations of naive CD8 T cells in aged mice, formally establishing a link between skewed spectratype profiles and reduced repertoire diversity. The detection of clonal expansions in the naive population of CD8 T cells is a novel observation indicative of age-associated perturbations in mechanisms of homeostatic proliferation.

These data are consistent with previous reports describing an age-associated loss of repertoire diversity in naive human T cells. In one study, spectratype analysis of naive peripheral human CD4 T cells suggested highly restricted oligoclonal T cell repertoires (29). In other studies, analysis of repertoire diversity showed a dramatic decline after age 70 (16), and naive T cells from elderly individuals >65 years of age were shown to have highly skewed spectratype profiles (55). Our data confirm and extend these observations for naive CD8 T cells from aged mice. Importantly, we have formally established a link between skewed spectratype profiles and clonal expansions. Whereas there was no evidence of clonal expansions in spectratype peaks from young mice or unskewed peaks from aged mice, at least some peaks from skewed, aged spectratype profiles were shown to contain clonal expansions.

These data support the premise that with decreased thymic export associated with aging, maintenance of the pool of naive T cells by homeostatic proliferation results in reduced repertoire diversity. In further support of this scenario, it has previously been shown that there is increased turnover of naive T cells with aging (16, 56). We propose that the clonal expansions we have identified in naive T cells are a direct consequence of homeostatic proliferation in an environment of severely reduced thymic export. Repertoire diversity has thus become compromised because of pressures to maintain the size of the pool (18).

Our data raise the question of how clonally expanded cells can maintain a naive phenotype. The response to survival signals results in homeostatic proliferation but not in activation; therefore, naive T cells undergoing homeostatic proliferation typically do not express cell surface markers associated with activation, such as CD44 (57). Naive T cells can transiently acquire a memory-like phenotype during homeostasis-driven proliferation, but show no change in other activation markers, such as CD62L, CD25, or CD49d and, in a normal, nonlymphopenic environment, the cells revert to a $CD44^{low}$ phenotype after homeostatic proliferation (58–60). Thus, although homeostatic proliferation may sometimes be associated with up-regulation of activation/memory markers (CD44), this is typically followed by reversion to a naive phenotype. An exception has been reported in extreme cases such as intense homeostatic proliferation associated with repopulation of an empty host in which it has been shown that naive T cells can permanently convert to a $CD44^{high}$ phenotype (60). It has also been suggested that intense homeostatic proliferation of naive cells in the elderly, perhaps to a self-peptide, may serve to drive naive T cells into the memory pool, further contributing to the shift in the naive to memory phenotype ratio (56).

We find no evidence for a consistent relationship between the skewed repertoire in naive and memory CD8 T cells from individual aged mice. This argues against both a mechanism by which clonally expanded naive CD8 T cells are driven into the memory pool and a mechanism by which clonally expanded memory cells lose their memory phenotype and inappropriately appear to be naive. Thus, the data support our hypothesis that the expansions we see in the naive pool are a consequence of dysregulated homeostatic proliferation. Failure to find evidence for crossover between the naive and memory pools re-enforces the idea that the naive and memory cells occupy separate niches in the periphery and are regulated independently.

The development of clonal expansions suggests a selective mechanism for preferential homeostatic proliferation of specific clones. Individual T cell clones may have different requirements for their maintenance, probably depending on TCR avidity and the availability of selecting ligands (57, 61). In adoptive transfer experiments, it was shown that in the absence of cognate Ag there was differential proliferation among transferred T cells. In addition, there was a hierarchy of thymic and peripheral selection in which some lymphocytes were more easily replaced by competitors than others. As mentioned above, in the elderly, preferential homeostatic proliferation may be largely driven by self-Ag, resulting in the development of autoreactivity. In support of this idea, it has been shown that as thymic output declines, the number of self-reactive T cells that may invade the peripheral pools increases (14). This is further supported by emergence of autoimmune diseases after thymectomy and the age-related increase in self-reactive lymphocytes (62, 63).

An important issue, especially with regard to therapeutic intervention and attempts to restore repertoire diversity, is to determine

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the kinetics of the age-associated decline of repertoire diversity. In humans, there is a major involution of the thymus during adolescence and thymic output declines dramatically between 25 and 60 years of age. However, cycling remains steady, the proportions of naive and memory T cells are maintained beyond age 65, and loss of repertoire diversity does not become apparent until approximately age 75 (16). Eventually, the reduced rate of thymic export cannot adequately replenish the peripheral pool and maintenance of pool size becomes almost completely dependent on homeostatic proliferation. Thus, after age 70, homeostatic proliferation doubles, repertoire diversity contracts dramatically, and the phenotypic distinction between naive and memory CD4 T cells becomes unclear (16). In our study, kinetic analyses were not performed. Our analysis of individual 19-mo-old mice showed that at this age the mice exhibit aged repertoires in terms of naive/memory distribution and the impaired ability to respond to influenza virus, yet have not developed a significant degree of clonal expansions in the memory pool (11) (data not shown). It will be important to determine at what age mice show evidence for loss of diversity among naive T cells in terms of skewed spectratype profiles and clonal expansions.

Age-associated declining repertoire diversity of naive CD8 T cells is thought to correlate with impaired immunity (7–11, 64). In support of this, we have previously shown an age-associated decline in reactivity to influenza virus-specific epitopes of low naive precursor frequency and restricted diversity. In some cases, the decline in repertoire diversity was so extreme that it resulted in "holes in the repertoire" of aged mice to normally immunodominant epitopes. Importantly, the age-associated decline in repertoire diversity and loss of reactivity to the immunodominant epitope correlated both with impaired cellular immunity to de novo influenza virus infection and a compromised recall response to heterologous infection (11). More recently, it has become possible to accurately determine the precursor frequency of naive CD4 and CD8 T cells of a given specificity. In one study, the number of peptide-specific CD4 precursors ranged between 20 and 200 (65) and, in another study, the number of peptide-specific CD8 precursors ranged between 80 and 1200 cells per mouse (66). Importantly, the CD4 precursor frequency predicted the size and TCR diversity of the primary CD4 T cell response following peptide immunization. The CD8 study showed that the Vβ bias in responding CD8 T cells was determined by a bias in precursor frequency and that the initial precursor frequency regulated the kinetics and immunodominance of the primary response and also controlled the development of the memory population. Whereas age-associated loss in diversity may not always result in a "hole" in the repertoire, as in our previous studies (11), it is now possible to formally study the impact of age-associated declines in repertoire diversity to particular epitopes on responsiveness in individual mice.

In conclusion, at least one contributing factor to the well-characterized age-associated decline in immune function is reduced naive T cell repertoire diversity due to loss of thymic function. Thus, an important goal is the development of treatments to maintain or restore the size of thymus and/or to enhance thymic export in elderly individuals (67–69). A key point is whether, with restoration of high thymic output, the diversity of the repertoire can be restored (70, 71). A variety of therapeutic approaches are being tested, including thymic transplants (72) and restoration of thymic progenitor cells (73, 74) or hematopoietic stem cells (75). In addition, methods such as chemical or physical castration to lower circulating levels of sex hormones (76–78) or treatment with IL-7 alone, or in combination with LHRH agonists (67, 79, 80), have been shown to increase the rate of export from the existing, but atrophied, thymus. Finally, growth hormones to increase thymic mass (81) should be tested, as it has been shown that thymic output correlates with the size of the thymus and is independent of age (22). The mouse provides a good experimental model for determining whether T cell repertoire diversity can be restored in the aged and whether this will result in improved immunity to new infections and vaccines.

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Disclosures

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