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Two Compensatory Pathways Maintain Long-Term Stability and Diversity in CD8 T Cell Memory Repertoires\textsuperscript{1,2}

Elena N. Naumova,\textsuperscript{*} Jack Gorski,\textsuperscript{†} and Yuri N. Naumov\textsuperscript{3,‡}

The time-dependent changes of human memory T cell repertoires are still poorly understood. We define a T cell memory repertoire as the pool of clonotypic lineages participating in a recall response to the influenza M1\textsubscript{58–66} epitope. In HLA-A2 individuals, this response predominantly uses BV19 chains with Arg-Ser (RS) in the CDR3 loop. We previously showed that the repertoire is polyclonal with a large fraction of clonotype that are only observed once. In this study, we perform longitudinal analyses of memory repertoires in three middle-aged individuals at times that spanned from 7 to 10 years. In these individuals, who are well into thymic involution, a substantial number of clonotypes were stable, e.g., detected at two times. The shape of the repertoire was stable over time as reflected by a number of repertoire characteristics, including singletons, i.e., the fraction of clonotypes observed into thymic involution, a substantial number of clonotypes were stable, e.g., detected at two times. The shape of the repertoire was stable over time as reflected by a number of repertoire characteristics, including singletons, i.e., the fraction of clonotypes observed only once, and repertoire diversity. However, the RS-clonotype subset showed a significant decline in the fraction of singletons and stable over time as reflected by a number of repertoire characteristics, including singletons, i.e., the fraction of clonotypes observed only once, and repertoire diversity. However, the RS-clonotype subset showed a significant decline in the fraction of singletons and in clonotypic diversity. Thus, repertoire structure is maintained over time by a recruitment of non-RS-clonotypes and a shift of existing RS-clonotypes into higher frequencies. The recruitment of new clonotypes into the low-frequency component of the repertoire implies a role for these clonotypes. \textit{The Journal of Immunology,} 2009, 183: 2851–2858.

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\textsuperscript{2}The contents of this publication are solely the responsibility of the authors and do not represent the official view of the National Institutes of Health.

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\textsuperscript{4}Abbreviations used in this paper: RS, Arg\textsubscript{2}Ser\textsubscript{3}; GxY, Gly\textsubscript{2}X\textsubscript{3}Tyr\textsubscript{4}; CDR3\textsubscript{aa}, CDR3 amino acid.

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HLA-A2 individuals. It has been observed that aging individuals can display highly focused CD8 T cell repertoires (22, 23), and it might be possible to observe the focusing of this complex repertoire as individuals age. To study the change in T cell memory, we analyze temporal changes in different repertoire characteristics: clonotype distributions, clonotype stability, CDR3 amino acid usage, the extent of the low frequency tail, and a measure of repertoire diversity modeled on the concept of species diversity in ecology. We demonstrated that the memory repertoires remain diverse, and there is temporal clonotype stability. However, there is a measurable decrease in repertoire diversity over time when the RS-containing clonotypes are considered. This decrease is countered by recruitment of non-RS-clonotypes into the repertoire.

Materials and Methods

Donors

Three healthy male blood donors were identified as HLA-A2,1 by DNA typing. Blood samples were collected from donor 1 in 1996 and 2004 (age 38 and 48 years), donor 2 in 1999 and 2006 (age 35 and 43 years), and donor 3 in 1999 and 2006 (age 49 and 56 years, respectively). The study was approved by the Institutional Review Board of the BloodCenter of Wisconsin.

M15 8–6 6-specific CD8 T cell cultures

PBMC were isolated using Ficoll-Paque plus (Amersham Biosciences) according to the manufacturer’s recommendations. For initial stimulation, PBMC were seeded in triplicate cultures as 2 × 10^6 cells/ml, 2-ml total volume, in a 13-ml round-bottom tube. At day 1, M15 8–6 6 peptide was added to the cultures at 1 μM of final concentration in the complete RPMI 1640 medium (Mediatech) supplemented with 10% heat-inactivated AB serum (Atlanta Biologicals), 10 U/ml human rIL-2 (BD Biosciences), 1% t-glutamine (Mediatech), and 1% HEPES (Fisher Scientific). The culture was carried for 3 wk with adding rIL-2 every 3 days and restimulating at day 7. Restimulation used irradiated (3000 rad) autologous PBMC pulsed with peptide Ag, with a 3:1 ratio with the cultured cells. At day 21, cells were harvested and used for CD8 T cells isolation using Dynal CD8 Positive Isolation Kit (Invitrogen). The historical data generated from the 1994 blood sample from donor 1 (19) were from 5-wk cultures.

RNA isolation, cDNA synthesis, VB19 spectratyping, and CDR3β subcloning

Cells from peptide-stimulated cultures were used for RNA isolation and cDNA synthesis as described elsewhere (24). Briefly, 1 μg of total RNA extracted from cell cultures using TRIzol (Life Technologies) were converted in to cDNA samples in 50 μl of reaction mixture that contained 500 units of Moloney murine leukemia virus reverse transcriptase (24). Aliquots of cDNA synthesis mixtures (2, 1, and 0.5 μl) amplified with VB19- and FAM-labeled BV19 primers (30 cycles) were resolved on 5 M urea/5% polyacrylamide sequencing gels. After gel scanning on FluorImager 595 (Molecular Dynamics), the generated VB19 CDR3 spectratypes were quantified using ImageQuant (IQMac v1.2) software (images are not shown). The CDR3β bands, which aligned with CDR3β bands of M15 8–6 6 tetramer-positive cells (positive control (21)) and had liner increase of the amplification signals, were extracted from gels and soaked in distilled water overnight. Two microliters of DNA solutions was reamplified with VB19- and unlabeled CB primers (two cycles) and subcloned in pCR4-TOPO plasmid vector using TOPO TA cloning kit for sequencing (Invitrogen).

VB19-CDR3β plasmid isolation and cycle sequencing

The nucleotide sequencing of the VB19 cDNA libraries was performed in the Harvard Medical School–Partner Healthcare Center for Genetics and Genomics. Briefly, 192–384 single colonies from each of triplicate plated cDNA library were transfer in to 200 μl of LB-Amp medium using standard Agencourt SprintPrep protocol and further sequenced using BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems). Finally, cleaned products were electrophoresed on an ABI 3730xl DNA Analyzer. 

Repertoire enumeration. We define a repertoire as a set of T cells specific for the peptide Ag, with a 3:1 ratio of Ag to cells as a clonotype as a unique β-chain CDR3 nucleotide sequence arising from VDJ recombination. The peptide specificity can be defined by tetramer binding or by expansion or activation in an in vitro recall response; we use the latter to define Ag specific cells. We examine the number of responding T cells using the colony-counting approach used previously (19–21). Comparison of in vitro recall repertoires with a direct pentamer analysis on a number of samples from HLA-A2 individuals indicates very good correlation (M. Yassai et al., manuscript in preparation). Therefore, we used peptide stimulation of PBMC in 3-wk cultures to generate a sufficient number of epitope-specific cells for molecular analysis. Triplicate cultures were performed to assure significant sampling of low-frequency clonotypes, and aggregate data from triplicate cultures were used for analysis.

Data analyses. We denote the total number of observed clonotypes as N. The number of colonies containing the same sequence in all three cultures yields the clonotype frequency, which can vary from 1 to the highest value observed (R_{MAX}). Clonotypes observed once are referred to as singlets, and the number of singleton clonotypes observed is given as Ns. These two measures define the boundaries of the clonotype distribution in a repertoire. The total number of observations of the most abundant clonotype is denoted as M. The ratio of M and N is the average number of observations per clonotype (V = M/N). The two extreme scenarios are as follows: 1) a uniform polyclonal response, in which case each clonotype is only observed once, so M = N and V = 1; and 2) a monolonal response in which all observations are of the same clonotype, so N = 1 and V = M.

A repertoire can also be also viewed as an assembly of CDR3 amino acid (CDR3aa) sequences involved in epitope recognition. For this kind of enumeration, the total number of distinct CDR3aa sequences observed is denoted as L. The ratio of the total number of clonotypes (N) and the total number of CDR3aa sequences (L) is an average number of clonotypes per a sequence (V_{sa} ≈ NIL). We define the length of the CDR3 as the number of amino acids starting immediately after the conserved cysteine in the V region and ending with the position directly before the conserved FGxGT region.

Diversity measures. We define clonotype diversity in a repertoire by drawing on the similar notion of species diversity in a ecosystem, widely used in ecology (25). Species diversity incorporates two other concepts: species richness and species abundance. Species richness, or in our case clonotype richness, is a simple concept defined as the number of species in a system, or in our case as the number of clonotypes in a repertoire, N. Species abundance refers to the representation of species in a system. This is a concept that is linked to the concept of species richness. In our case, we focus on repertoire evenness, or more specifically to unevenness in the form of the number of observations of the most abundant clonotype, R_{MAX}.

Thus, our definition of clonotype diversity incorporates clonotype richness and clonotype abundance components. Because our clonotype counting procedure is not exhaustive, we correct the abundance measure R_{MAX} by the number of sequences in a sample, M. Thus, the ratio R_{MAX}/M serves as a proxy for species evenness. Before, we defined clonotype diversity as a composite of species richness and species evenness measures: N × (R_{MAX}/M). In the two extreme scenarios described above, this measure will be equal to 1: 1) if the repertoire is monoclonal, R_{MAX} = M and N = 1; and 2) if each clonotype is present once, N = M and R_{MAX} = 1. A biological system, which consists of only one species, and a system in which all species are equally abundant by definition are not considered diverse. Therefore, we use a normalized version of diversity measure, Dc = N × R_{MAX}/M − 1, to better reflect these extreme scenarios, so the diversity of repertoires under the two extreme conditions is zero. A species-rich ecosystem with absolute species evenness is not considered diverse, as diversity extends to the number and distribution of species.

Measuring temporal change. To examine temporal variations in memory repertoires, we estimated the ratio of change, as the absolute difference in the number of clonotypes involved in epitope recognition by the number of years lapsed between them. Finally, we tested if estimated rates are significant by a two-sided nonparametric test and presented corresponding p values.

CDR3aa sequence analysis. We describe the CDR3aa sequence composition of the repertoire on the basis of the amino acids and their frequency in each of four core positions, P1 through P4, with P4 being the position immediately following the second serine of the CASS sequence at the end of the VB19 gene. We use this approach to compare sequences with identical CDR3 length.

Online supplemental material

Supplemental Table 1 shows the B region identifier, CDR3β amino acid and nucleotide sequences, and number of observations of influenza A M15 8–6 6-specific VB19 clonotypes from donor 1 (in years 1994 and 2004), donor 2 (in years 1999 and 2006), and donor 3 (in year 1999 and 2006).

The online version of this article contains supplemental material.
The values shown within boxed regions correspond to the numbers of observations of clonotypes defined at both times. The characters in the CDR3aa sequences shown in bold indicate four core amino acids (P1-P2-P3-P4) involved in M1<sub>58–66</sub>/HLA-A2 recognition.

**Results**

*The general shape of the recall repertoire is conserved over time*

To examine the long-term evolution of M1<sub>58–66</sub>-specific repertoires, recall cultures from blood samples from three donors (donors 1–3) collected at different times were analyzed: cDNA was prepared from the cultured cells, and the BV19 CDR3 were amplified by PCR. cDNA products of 159 and 156 nt (CDR3β of 11 and 10 aa residues, respectively) were subcloned into plasmid vectors and sequenced. The details of the sequence analysis of the M1<sub>58–66</sub>-specific repertoire identifying the clonotypes and the number of times they were observed can be found in supplemental Table I. Table I summarizes the primary characteristics of the repertoires: the number of unique clonotypes (N); the number of sequences analyzed (M); the number of observations of the most frequent clonotype(s) (R<sub>MAX</sub>); the number of singletons, (Ns); and the number of different CDR3 amino acid sequences (L) for each donor at each time.

The clonotype frequencies plotted in descending order for each individual at each time point (Fig. 1) show a clear similarity in clonotype distributions. Each distribution contains a few clonotypes of high frequency, a number of middle-range frequency clonotypes, and a “long tail” of singletons. The general shape of the repertoire is conserved among individuals and over time.

*A major fraction of the influenza-specific VB19 repertoires is stable*

The clonotype frequency distributions shown in Fig. 1 represent a general description of the repertoires at different times but do not show whether individual clonotypes appear once or at both times. To illustrate the extent to which any clonotype is observed at a particular time point, clonotype distributions that combine the two time points are shown for each donor, using color to show the contribution from each time (Fig. 2). The insets show the clonotypes observed 10 or more times and identify the amino acid sequence of the clonotype. It can be seen that some clonotypes were observed at both times (i.e., two colored bars) and are referred to as stable clonotypes. The fraction of stable clonotypes for each donor is shown in Fig. 2 and row 1 in Table II. The number of stable clonotypes varied between 15 and 30%.

The combined clonotype distributions shown in Fig. 2 have a long tail of clonotypes only observed once, i.e., at one time or the other. Because their low level of sampling precludes their being observed at both times, it can be argued that these clonotypes should not be considered in calculating the proportion of the repertoire that is stable. If we exclude those clonotypes only observed once and consider the data from clonotypes observed two or more times, the clonotype stability doubles (row 2 in Table II). Because stability is a function of the probability of observing the clonotype, we estimated the fraction of stable clonotypes with respect to the combined distributions for each donor and for six thresholds of clonotype frequency. Table II illustrates the increase in stability when we consider the six nonexclusive subsets of clonotypes observed in the combined data more than one, two, three, four, five, and six times. For clonotypes observed four or more times in the combined dataset, stable clonotypes represent >50% of the repertoire for all three individuals (Table II). For clonotypes observed six or more times, >60% of the clonotypes are stable. This indicates that temporal stability is related to our ability to measure the repertoires in detail and that the clonotypes are generally stable.

**Clonotype diversity is stable, yet CDR3β amino acid sequence utilization increases with aging**

As part of our repertoire analysis, we calculated repertoire characteristics that provide insight into clonotypes distributional properties (Table III). The average number of observations per clonotype (V = M/N) provides an overall measure of the number of clonotypes observed at both times and number of clonotypes at cumulative frequency and higher.

![FIGURE 1. Clonotype distributions for the three donors. The donors are identified on the x-axis. The year blood was collected is identified above each clonotype distribution. The number of observations of each clonotype is on the y-axis. The clonotypes identified for each donor at each time are plotted on the x-axis in decreasing order from the back. The singletons represent clonotypes observed only once, and high-frequency clonotypes are defined as those observed 10 or more times.](http://www.jimmunol.org/)

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**Table I. Repertoire measures for each donor at both sample times**

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of clonotypes, N</td>
<td>135</td>
<td>97</td>
<td>59</td>
<td>63</td>
<td>52</td>
<td>97</td>
</tr>
<tr>
<td>No. of observations, M</td>
<td>494</td>
<td>673</td>
<td>531</td>
<td>804</td>
<td>369</td>
<td>573</td>
</tr>
<tr>
<td>Maximum no. of observations per a unique clonotype, R&lt;sub&gt;MAX&lt;/sub&gt;</td>
<td>81</td>
<td>98</td>
<td>266</td>
<td>210</td>
<td>101</td>
<td>90</td>
</tr>
<tr>
<td>No. of singletons, Ns</td>
<td>80</td>
<td>51</td>
<td>24</td>
<td>25</td>
<td>23</td>
<td>62</td>
</tr>
<tr>
<td>No. of CDR3β amino acid sequences, L</td>
<td>58</td>
<td>57</td>
<td>31</td>
<td>35</td>
<td>32</td>
<td>79</td>
</tr>
</tbody>
</table>

---

**Table II. Stability as a function of clonotype frequency**

<table>
<thead>
<tr>
<th>Cumulative Frequency</th>
<th>Donor 1</th>
<th>Donor 2</th>
<th>Donor 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15% (30/202)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30% (28/94)</td>
<td>16% (21/128)</td>
</tr>
<tr>
<td>2</td>
<td>34% (30/88)</td>
<td>46% (28/61)</td>
<td>39% (21/54)</td>
</tr>
<tr>
<td>3</td>
<td>47% (26/55)</td>
<td>50% (25/50)</td>
<td>53% (18/34)</td>
</tr>
<tr>
<td>4</td>
<td>57% (24/42)</td>
<td>55% (24/44)</td>
<td>58% (15/26)</td>
</tr>
<tr>
<td>5</td>
<td>58% (21/36)</td>
<td>53% (23/43)</td>
<td>58% (15/26)</td>
</tr>
<tr>
<td>6</td>
<td>61% (20/33)</td>
<td>65% (22/34)</td>
<td>63% (15/24)</td>
</tr>
</tbody>
</table>

<sup>a</sup> The number of observations made independent of the sample.

<sup>b</sup> Percentage of stable clonotypes at the given cumulative frequency and higher.
clonotypes corrected for sample size and is analogous to species richness in ecology. The average number of clonotypes per CDR3aa sequences \( \left( V_{aa} = N/L \right) \) does the same for amino acid sequences represented in the repertoire. The fraction of singletons describes the tail of the repertoire. The clonotype diversity measure \( (Dc = N \times R_{\text{MAX}}/M - 1) \) incorporates the distributional effect of the clonotype present at highest frequency. The rate of change of repertoire measures per year, \( \Delta \), allows pairwise comparisons of repertoire changed using nonparametric \( t \) test. The only change over time that reached statistical significance was the average number of clonotypes per CDR3aa sequence \( \left( V_{aa} = N/L \right) \). The decrease in this measure signifies an increase in the number of different amino acid sequences \( (L) \) relative to the number of clonotypes, either because the \( L \) went up or the \( N \) went down.

**CDR3β amino acid utilization changes with age**

To further investigate the significance of the decrease in \( V_{aa} \), we examined any changes that may be taking place in the generally constrained BV19 CDR3aa utilization of this response. We plotted the CDR3aa utilization on a position-by-position basis across the four core positions \( (P_1-P_2-P_3-P_4) \) to determine whether there is a difference with time. Such an analysis can only be performed of CDR3 of the same length. Thus, the data shown are restricted to the majority repertoire component, clonotypes with a CDR3 length of 11 aa (Fig. 3). The amino acids with the highest frequencies at each position are highlighted and represent the dominant sequence, IRSS. Amino acids that were present at both time points (overlap) are boxed. In donor 1 (Fig. 3A), the number of amino acids

### Table III. Repertoire characteristics and their rate of change

<table>
<thead>
<tr>
<th>Repertoire Characteristics</th>
<th>Donor 1</th>
<th>Donor 2</th>
<th>Donor 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1994</td>
<td>2004</td>
<td>( \Delta^a )</td>
</tr>
<tr>
<td>Average number of observations per clonotype, ( V = M/N )</td>
<td>3.66</td>
<td>6.94</td>
<td>-0.33</td>
</tr>
<tr>
<td>Average number of clonotypes per CDR3aa sequence, ( V_{aa} = N/L )</td>
<td>2.33</td>
<td>1.70</td>
<td>0.06(^b)</td>
</tr>
<tr>
<td>Fraction of singletons (percentage of all clonotypes)</td>
<td>59</td>
<td>53</td>
<td>0.67</td>
</tr>
<tr>
<td>Clonotype diversity, ( Dc = R_{\text{MAX}} \times N/M - 1 )</td>
<td>21.14</td>
<td>13.12</td>
<td>0.80</td>
</tr>
</tbody>
</table>

\(^a\) Rate of change per year.

\(^b\) Characteristics exhibiting significant changes are shown in bold.
observed in positions $P_2$ and $P_3$ increased with age. In donor 2 (Fig. 3B), amino acid usage increased at all position except $P_4$. For donor 3 (Fig. 3C), there was an increase in amino acid usage in three of the four positions. This change was also evidenced in the sum of the relative frequencies of the overlapping amino acids (Fig. 3A–C, boxed for each donor).

To minimize the individual variations of the CDR3 usage among individuals, we calculated the average sum of frequencies of the overlapping amino acids for all donors (Fig. 3D, boxed). There was a decrease in the overlapping amino acids in the CDR3.

These data point to a reduced usage of the amino acids that constitute the RS motif.

**Decrease in the diversity of RS-clonotypes with age**

The data from Table IV and Fig. 3 indicate a change in the number of RS-clonotypes. We therefore repeated our previous analysis on this major repertoire subset. In our datasets, the fractions of the RS-clonotypes varied from 29 to 80% of all defined clonotypes (Table IV, row 1). The number of RS-clonotypes decreased with age in donors 1 and 3.

We examined the stability of the RS-clonotypes in a similar manner to that presented in Table II and observed that RS-clonotypes are stable to the same extent as all clonotypes are stable (data not shown).

The primary repertoire measures for the RS-clonotypes are shown in Table IV. There were significant changes in three of the four calculated characteristics of the RS-clonotypes (Table V). There was a 2-fold increase in the average number of sequences per clonotype ($p < 0.03$) or a loss of about one copy per clonotype per year. There was a significant reduction in the fraction of singletons ($p < 0.05$). This is equivalent to the loss of one unique clonotype out of 100 detected singletons per year. Finally, the RS-clonotype diversity, a measure incorporating the contribution of the most frequently observed clonotype ($R_{\text{MAX}}$) and the number of clonotypes ($N$), declined in all three donors ($p < 0.05$). The lack of significance in changes to $V_{\text{aa}}$ was expected because we are analyzing a repertoire subset defined by CDR3aa sequence.

We plotted the changes in the repertoire characteristics that showed significant changes over time as a function age of the individual (Fig. 4). There is no evident relationship between age and clonotype richness as measured by $V$ (Fig. 4A). The initial values appear to be specific for the individual but independent of age. The increase in $V$ appears to be specific to each individual as evidenced by the different slopes. There is also a lack of an evident relationship between age and the fraction of singletons (Fig. 4B). However, there is evidence for a relationship between age and repertoire diversity (Fig. 4C). There is a trend toward colinearity of the all the points. This indicates that a diversity measure that incorporates both repertoire evenness and richness may be a better descriptor of changes in a population over time.

**Overall changes in the repertoire with time**

The above analyses indicated the importance changes in repertoire measures of subsets of the responding repertoire. To obtain a general description of changes in the repertoire over time, we measured the relative contribution of subsets based on: CDR3 length of 11 aa, clonotypes with the CDR3aa motifs (-RS-), and clonotypes encoding IRSS. Each of these subsets is nested in the previous one. The percentage of clonotypes in each subset reflects the average of the data from the three donors for that subset. The change was measured in the overall repertoire as well as in distributional subsets: low-frequency (1–2 observations), mid-frequency (3–10 observations), and high-frequency clonotypes (>10 observations) (Fig. 5).
expressing this CDR3aa sequence are more stable than the broader -RS- subset.

The reduction in the RS subset was predominantly driven by the low-frequency component of the repertoire that represents singlets and doubletons (Fig. 5B). The singlets contributed just over 70% of this decrease with the remaining contribution from the doubletons. There was a very moderate decrease in the mid-frequency component defined by clonotypes observed 3–10 times (Fig. 5C). The decrease in the low-frequency component of RS-clonotypes is somewhat compensated by an increase in the high-frequency component, which is defined by clonotypes observed >10 times (Fig. 5D). Thus, the summary of overall changes is a decrease in RS-clonotypes as a percentage of clonotypes with CDR3 of 11 aa and a shift toward higher frequency distributions.

Discussion

Clonal expansion and contraction of the T cells specific to a particular epitope play an important role in generating a memory repertoire. The cytotoxic repertoire we analyze here is most likely due to recurrent pathogen-host interactions as the response to M1 protein, conserved in all human influenza A strains. It would be boosted with each exposure, whether clinical or subclinical. We proposed that recurrent exposures lead to a period of 7–10 years in individuals where the arrival of new clonotypes is reduced due to thymic involution. To achieve this goal, we examined recall repertoires of the well-studied HLA-A2-restricted response to influenza M15 8–6 6 in three individuals at two time points. The T cells that constitute a line that has been stimulated three times are generally considered to be Ag specific. We generated clonotypic data for VB19 PCR and analyzed the repertoires in these lines. The restriction of our analysis to VB19 reflects the predominant use of the V family in this response (10, 11, 18). There is an occasional use of other VB families, but these constitute a low percentage of the overall response (12). In other analyses, we find that the percentage of VB19-positive cells that are also positive for HLA-A2:M15 8–6 6 tetramer binding can vary from 90 to 99% (21). Thus, it is possible that a small proportion of the clonotypes studied here might not be able to bind tetramers. The significance of this to the specificity of the clonotypes is a function of the extent to which tetramer binding reflects a functional response.

By plotting clonotype frequency distributions, we observed that the overall shape of the clonotype distribution was maintained over time in all three individuals (Fig. 1). In keeping with the model that middle-aged repertoires are characterized by long-lived clonotypes, we observed that a large proportion of the clonotypes in the repertoires are stable in all three individuals. The exact proportion varies based on the assumptions as to what proportion of the clonotypes contains a low percentage of the overall response (12). In other analyses, we find that the percentage of VB19-positive cells that are also positive for HLA-A2:M15 8–6 6 tetramer binding can vary from 90 to 99% (21). Thus, it is possible that a small proportion of the clonotypes studied here might not be able to bind tetramers. The significance of this to the specificity of the clonotypes is a function of the extent to which tetramer binding reflects a functional response.

Although the general repertoire shape stays the same, and many clonotypes are stable, there are changes that occur with time. One of these changes is in the CDR3aa utilization of clonotypes making up the repertoire. The response to M15 8–6 6 in HLA-A2 individuals has been shown to use a restricted number of amino acids, especially in the second and third positions (10, 11). In general, the use of other amino acids at all four positions increases with age. These changes, together with a decrease in the number of clonotypes encoding the same CDR3aa sequence, indicated possible changes in the subset of clonotypes containing RS. Therefore, we examined repertoire characteristics over time in this subset.

Table IV. RS-repertoire measures

<table>
<thead>
<tr>
<th>RS-Clonotypes Repertoire Measures</th>
<th>Donor 1</th>
<th>Donor 2</th>
<th>Donor 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of observations, M</td>
<td>266</td>
<td>210</td>
<td>64</td>
</tr>
<tr>
<td>Maximum number of observations per a unique RS-clonotype, R_{MAX}</td>
<td>81</td>
<td>98</td>
<td>55</td>
</tr>
<tr>
<td>No. of RS-singletons, Ns</td>
<td>64 (59%)</td>
<td>24 (44%)</td>
<td>20 (47%)</td>
</tr>
<tr>
<td>No. of RS-CDR3β amino acid sequences, L</td>
<td>36 (62%)</td>
<td>20 (35%)</td>
<td>18 (58%)</td>
</tr>
</tbody>
</table>

Table V. RS-repertoire characteristics and their rate of change

<table>
<thead>
<tr>
<th>Repertoire Characteristics</th>
<th>Donor 1 2004</th>
<th>Δ</th>
<th>Donor 2 2006</th>
<th>Δ</th>
<th>Donor 3 2006</th>
<th>Δ</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average number of observations per clonotype, V = M/N</td>
<td>3.78</td>
<td>-0.57</td>
<td>10.21</td>
<td>-0.91</td>
<td>6.79</td>
<td>-0.38</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Average number of clonotypes per CDR3aa sequence, V_{aa} = N/M</td>
<td>3.00</td>
<td>2.70</td>
<td>3.93</td>
<td>2.65</td>
<td>-0.04</td>
<td>3.11</td>
<td>2.55</td>
</tr>
<tr>
<td>Fraction of singletons among RS-clonotypes (%)</td>
<td>59</td>
<td>44</td>
<td>150</td>
<td>47</td>
<td>31</td>
<td>2.29</td>
<td>36</td>
</tr>
<tr>
<td>Clonotype diversity, Dc = R_{MAX} \times N/M − 1</td>
<td>20.44</td>
<td>9.32</td>
<td>11.11</td>
<td>25.05</td>
<td>11.68</td>
<td>1.91</td>
<td>8.43</td>
</tr>
</tbody>
</table>

*Rate of change per year.
Characteristics exhibiting significant changes are shown in bold.
Changes in the RS-clonotype repertoire with age

The RS-clonotypes in all three donors showed a decrease in three calculated clonotype measures. The first of these, clonotype diversity, is a measure incorporating clonotype evenness and richness. Repertoire diversity is still a poorly defined concept, and although a standardized measure of diversity would allow comparisons of different studies, the current stage of our understanding of the generation, maintenance, and decay of an immune responses requires exploration of various approaches. We preferred not to limit ourselves to a definition of diversity based on a number of observed clonotypes (28), which emphasize repertoire richness, or of focusing on evenness (29). Because of the complexity of the notion of diversity, it is unlikely to be fully depicted by a single number. Undoubtedly, our formulation will be subject to future changes, but as it currently stands, evenness is represented by the $R_{MAX}$:M ratio, and richness by the number of clonotypes. The second measure is the proportion of singletons. This is a straightforward measure that did not change significantly in the overall repertoire. Combined, these two observations indicate that with time the singleton tail in the overall repertoire is composed of more clonotypes that do not have RS in the CDR3 loops. It should be pointed out that these two characteristics, the proportion of singletons and clonotype diversity, incorporate the two extreme measures of the repertoire. Finally, the proportion of RS-containing CDR3aa sequences decreased significantly in each individual.

A more global view of the repertoire changes showed that in addition to an increased number of non-RS-clonotypes in the low-frequency component, the overall repertoire shape is maintained by a shift in RS- and IRSS-clonotypes into the high-frequency component. The fact that this shift into higher frequencies is not mirrored by a significant increase in $R_{MAX}$ indicates that this shift is a population effect and not due to increases in a single clonotype.

Significance of age-related changes in RS-clonotypes

We propose that the division of the repertoire into a RS-clonotype subset and a subset of clonotypes without this motif could reflect two different mechanisms by which the recall repertoire is established. We propose that RS-clonotypes are established during the time of full thymic production. These clonotypes constitute a large stable component of the repertoire. However, by middle age their number starts to decrease. We propose that the second component to the repertoire we measure in recall responses, the subset without this motif, is recruited into the responding repertoire to maintain an optimum level of diversity commensurate with health. In the event of influenza exposure, or perhaps even vaccination, recruitment of clonotypes that do not use the RS CDR3 sequence would reinforce the core repertoire.

FIGURE 4. RS-repertoire characteristics as a function of age. A. Average number of observations per clonotype with RS in the CDR3β. B. Fraction of singletons within RS-repertoires (as percentage of RS-clonotypes). C. Clonotype diversity of RS-repertoires. The x-axis indicates the donor age at time of sampling.

FIGURE 5. Temporal changes in the relative contribution of repertoire subsets to the whole repertoire. Data are the average value for each subset from all three subjects. A. The entire repertoire is divided into the following nested subsets: clonotypes of CDR3 length 11 aa, clonotypes using the RS motif, and clonotypes encoding IRSS. B–D. Analysis of the three subsets on the basis of clonotype frequency. B. Clonotype observed once or twice. C. Clonotype observed 3–10 times. D. Clonotypes observed >10 times. The magnitude and direction of observed changes are indicated by arrows. The numbers indicate percent of clonotypes within each nested subset.
All three donors show stable repertoire characteristics with age, while at the same time the RS-clonotype subset is changing. All three donors show a change in the "non-RS" compartment with time to compensate both for the loss in diversity and the loss of the singletons. To the extent that the RS-clonotypes may represent the optimal responders to the M1\textsubscript{58–66} epitope, it is interesting that the immune system is capable of substituting other clonotypes as the RS-clonotype number diminishes. The substitution of possibly suboptimal clonotypes in the recall repertoire may speak to a more complex immunostasis mechanism than has been acknowledged to date.

The role of non-RS-clonotypes with a CDR3 length of 11 in responding to M1\textsubscript{58–66} has not yet been clearly established. Currently, there are no data directly associating non-RS-clonotypes and function. In our population analyses of HLA-A2 individuals, we often observe clonotypes with the RA sequence, and several of these are seen in the three individuals studied here. In a population, there are some individuals with higher percentages of non-RS-clonotypes, as is seen for donor 2 in this study. In our population studies, we have observed non-RS-clonotypes in M1\textsubscript{58–66} pentamer-selected T cells. In an individual with a higher proportion of non-RS-clonotypes, we observed a slightly higher proportion of non-RS-clonotypes (31%) in non-selected cultures than in pentamer-selected cultures (25%). Thus, it is possible that some of the non-RS-clonotypes observed may not have sufficient affinity to bind tetramers.

In light of the predominance of RS-clonotypes in the responding repertoire, it will be very interesting to determine how soon it is established and if exposure to influenza in middle age results in repertoire shifts involving novel clonotype recruitment. The source of these novel clonotypes is also an interesting question. In light of reduced thymic output, it is possible that such clonotypes are recruited from pre-existing memory to other pathogens with related epitopes. Such studies will require more careful longitudinal analyses with comprehensive testing for exposure during influenza season.

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Disclosures

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References