

# Diversity and clonal selection in the human T-cell repertoire

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**T-cell receptor (TCR) diversity, a prerequisite for immune system recognition of the universe of foreign antigens, is generated in the first two decades of life in the thymus and then persists to an unknown extent through life via homeostatic proliferation of naïve T cells.** We have used next-generation sequencing and non-parametric statistical analysis to estimate a lower bound for the total number of different TCR beta (TCRB) sequences in human repertoires. We arrived at surprisingly high minimal estimates of 100 million unique TCRB sequences in naïve CD4 and CD8 T-cell repertoires of young adults. Naïve repertoire richness modestly declined two- to fivefold in healthy elderly. Repertoire richness contraction with age was even less pronounced for memory CD4 and CD8 T cells. In contrast, age had a major impact on the inequality of clonal sizes, as estimated by a modified Gini-Simpson index clonality score. In particular, large naïve T-cell clones that were distinct from memory clones were found in the repertoires of elderly individuals, indicating uneven homeostatic proliferation without development of a memory cell phenotype. Our results suggest that a highly diverse repertoire is maintained despite thymic involution; however, peripheral fitness selection of T cells leads to repertoire perturbations that can influence the immune response in the elderly.

adaptive immune responses | aging | immunosenescence |  
T-cell homeostasis

The ability of the adaptive immune system to respond to a wide variety of pathogens depends on a large repertoire of unique T-cell receptors (TCRs). TCR diversity is generated by the random and imprecise rearrangements of the V and J segments of the TCR alpha (TCRA) and V, D, and J segments of the TCR beta (TCRB) genes in the thymus. Thymic production of T cells is the sole mechanism to generate TCR diversity. With the involution of the thymus with age, the generation of new naïve T cells with new TCRs dwindles (1, 2). In the mouse, residual thymic activity provides an ongoing source of naïve T cells, although not sufficient to maintain the compartment size. In contrast, homeostatic proliferation of peripheral T cells is the predominant mechanism of T-cell generation in the human adult (3).

Given the dramatic decline in thymic T-cell production, thymic involution has been implicated in defective T-cell responses with aging (4). Elderly individuals are more prone to develop complications from infections; in particular, they are susceptible to newly arising infectious organisms such as West Nile fever or severe acute respiratory syndrome (5, 6). Moreover, immune responses to vaccination are dramatically reduced (7). Shrinkage in the size of the naïve T-cell compartment and holes in the repertoire have been discussed as potential causative factors in age-related impaired adaptive immunity (8). Indeed, holes have been identified in the murine repertoire to contribute to the increased morbidity from influenza infection (9, 10).

Pioneering early work on estimates of diversity of the human TCR repertoire was based on sequencing a few hundred sequences and extrapolating to the scale of the entire repertoire, yielding an

estimated lower limit estimate of fewer than 1 million different TCRB genes (11). Studies in more recent years have used high-throughput sequencing to base estimates on greater sequencing depth. Warren et al. (12) measured 1 million different TCRB sequences in a peripheral blood sample. Robins et al. (13) inferred the number of unseen TCRB genes from deep sequencing data by applying a Poisson process model and estimated a diversity of 3–4 million TCRB sequences in the total T-cell populations of two healthy donors.

Although high-throughput DNA sequencing provides the tool to gather extensive datasets, estimates of TCR richness remain a challenge because clinical samples represent a small fraction of the  $10^{12}$  cells in the overall T-cell compartment. Richness, the actual number of unique TCR sequences in a T-cell population, is dominated by the infrequent species that are observed rarely or not at all. The major factors affecting accurate identification of rare species in TCR repertoires are the number of cells examined, the variable efficiency of PCR amplification, the expression levels of TCR transcripts in different T cells, and sequencing error rates. In addition, peripheral blood T cells consist of a mixed population of naïve, memory, and effector CD4 and CD8 cells that differ markedly in diversity, with memory populations being less complex than naïve populations.

## Significance

A decline in the diversity of the T-cell receptor repertoire owing to thymic involution has been implicated as causing defective immune responses in the elderly. By applying next-generation sequencing of replicate TCRB libraries from highly purified T-cell subsets, and using nonparametric statistical analysis, we obtain estimates of repertoire richness in the young adult that are higher than previously reported. Although contracting with age, the repertoire remains highly diverse. These data challenge the paradigm that thymic rejuvenation is needed to maintain diversity and prevent immune incompetence in the elderly. However, we observe an increasing inequality of clonal sizes with age even among naïve T cells. This clonal selection could result in biased and possibly autoreactive immune responses.

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Data deposition: The sequences reported in this paper have been deposited in the Sequence Read Archive and can be accessed via the database of Genotypes and Phenotypes (dbGaP), [www.ncbi.nlm.nih.gov/gap](http://www.ncbi.nlm.nih.gov/gap) (accession no. [phs000787.v1.p1](https://www.ncbi.nlm.nih.gov/gap/study.cgi?study_id=phs000787.v1.p1)).

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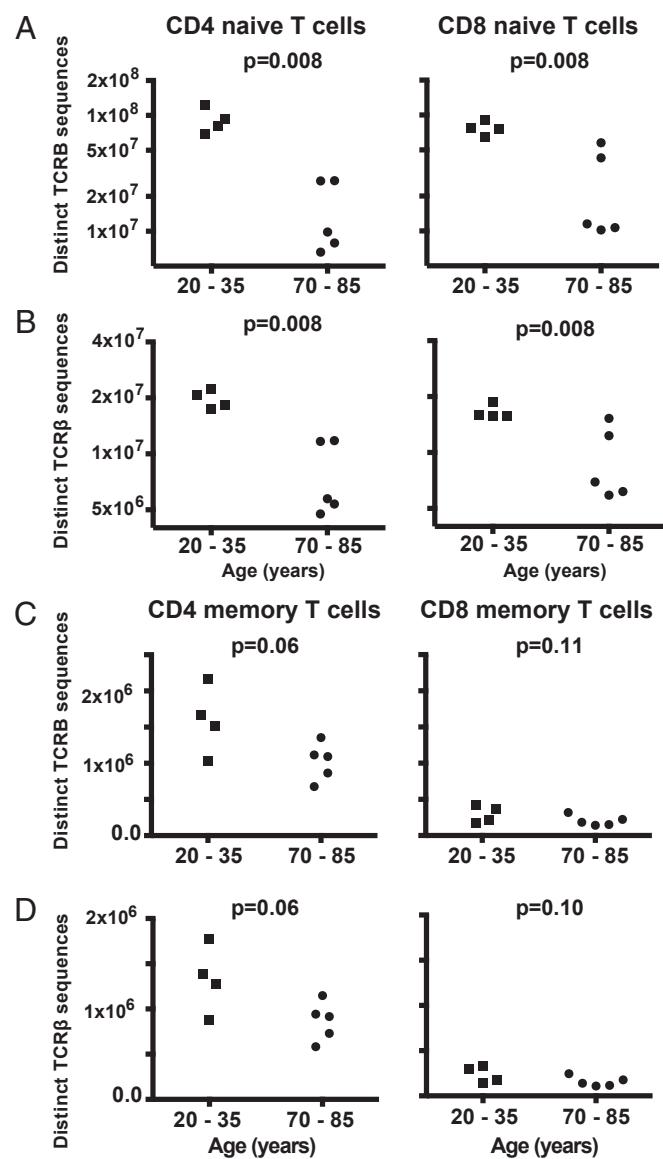
The current study was designed explicitly to improve estimation of the TCR repertoire richness of stringently defined naïve and memory T cells. By sequencing multiple replicate TCRB libraries of cells from each T-cell subset and applying nonparametric statistical analysis, we find that human TCR repertoires are an order of magnitude more diverse than previously estimated. Despite significant age-related decreases in richness, humans maintain high diversity during healthy aging. Strikingly, we find age-associated changes in the distribution of clonal sizes, in particular in the naïve compartment, which may reflect unevenness of homeostatic expansion with clonal expansion of some naïve T cells that equal or exceed clonal sizes of memory T cells. The inequalities in clonal sizes could compromise the immune response to the majority of antigenic epitopes while causing an increased responsiveness to selected few epitopes.

## Results

**Gene Segment Use and CDR3 Features in Young and Elderly TCRB Rearrangements.** In initially evaluating TCRB repertoires in young and elderly subjects we compared the composition of TCRB gene rearrangements at the level of TCRBV and TCRBJ gene segment use and the features of the CDR3-encoding junctional nucleotides. Apheresis lymphocytes were obtained from four 20- to 35- and five 70- to 85-y-old healthy adults who were regular platelet donors. Naïve CD4 and CD8 T cells were purified by cell sorting. We used a stringent definition of naïve cells ( $CD3^+CD4^+$  or  $CD8^+CCR7^+CD45RA^{high}CD28^+$ ) and very restrictive gating to ensure purity. Approximately 1.5–3 million sequence reads were obtained for each T-cell subset of each donor (Table S1). TCRBV and TCRBJ gene segments were used at comparable frequencies in the repertoires of naïve CD4 and CD8 T cells in young and old individuals (Fig. S1A), whereas the memory repertoires of CD4 and, particularly, CD8 T cells show variable and individual-specific gene segment use frequencies (Fig. S1B). CDR3 sequences in the young and elderly were comparable in length and did not show any definitive age-related features (Fig. S1 C and D).

**High Richness of Naïve CD4 and CD8 TCRB Repertoires in Young and Elderly Adults.** To obtain sequence data to estimate global TCRB repertoire richness we used the experimental design of analyzing a series of replicate libraries from independent cell aliquots from each T-cell subset in each individual. These replicates allowed us to calculate repertoire richness by applying the “Chao2” estimator, a nonparametric estimator of unseen species (14). The approach allows estimation of the extent to which the full repertoire is covered and use of this information to determine a lower bound of the total number of species in the repertoire. Because the Chao2 estimator requires only a binary characterization of presence or absence of each clone in each replicate library, it circumvents the challenges that arise in experimental designs using only a single library and avoids confusing the effects of PCR amplification with the presence of expanded T-cell clones. To not count possible sequencing errors as independent sequences, we rejected single sequences as erroneous if a highly similar clone of greater frequency was identified in the same library (see Materials and Methods for the definition of similarity).

The lower bounds on TCRB gene richness obtained with this approach yielded higher estimates than previous studies (Fig. 1). Young adults carried an estimated 60–120 million different TCRB genes, both in the CD4 and CD8 naïve T-cell repertoires. This high diversity in nucleotide sequences was reflected in a large functional repertoire of TCR  $\beta$  chains with a lower boundary of ~20 million different amino acid sequences. To determine the robustness of our estimates, we used two approaches to estimate confidence intervals. We applied the BC<sub>a</sub> variant of bootstrapping that is designed for obtaining confidence intervals when the underlying bootstrap distribution is not symmetric about its center

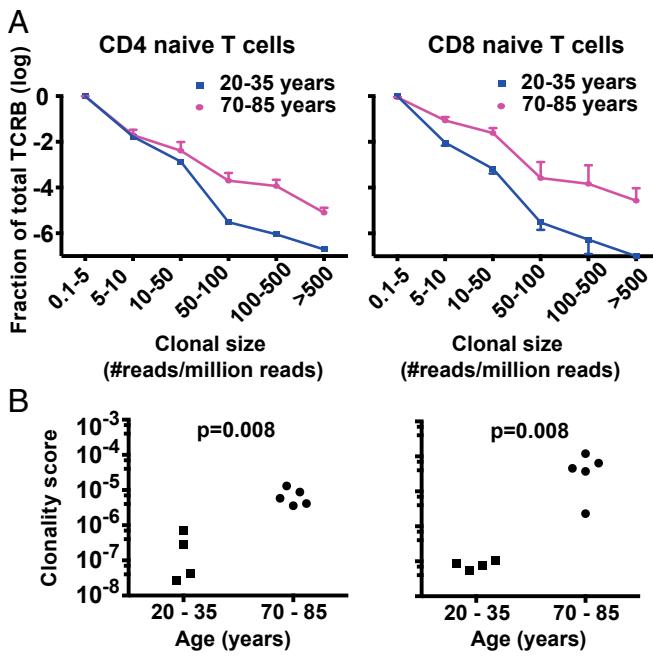


**Fig. 1.** Age is associated with a modest decrease in diversity of the TCRB repertoire. TCRB sequences were obtained from replicate samples of naïve (A and B) and memory (C and D) CD4 and CD8 T cells. A lower bound of TCRB richness was estimated by applying nonparametric statistics using the Chao2 estimator. Results are shown for nucleotide (A and C) and derived amino acid sequences (B and D). Estimates were compared by Wilcoxon–Mann–Whitney test. Increase in age is associated with a decline in richness of naïve CD4 and CD8 T cells; however, the repertoire in the elderly remains highly diverse. Richness in CD4 and CD8 memory T cells markedly differed, whereas the impact of age was negligibly small.

(15). Second, we estimated the confidence intervals using the approach originally developed by Chao (16). The 95% confidence intervals with both methods were very narrow (Table S2).

Naïve TCRB repertoire richness declined significantly in the 70- to 85-y-old adults to a lower bound richness of 8–57 million different nucleotide sequences encoding ~5–15 million TCR  $\beta$ -chain amino acid sequences ( $P = 0.008$ , Fig. 1 A and B). Interestingly, the estimates in elderly CD4 and CD8 naïve T cells were similar despite the greater decline in CD8 compared with CD4 naïve T-cell numbers with aging (17, 18).

**CD4 and CD8 Memory T Cells Differ in TCRB Richness Independent of Age.** Memory cells have been selected from the naïve repertoire and clonally expanded in response to antigen and are therefore



**Fig. 2.** Increase in clonal expansions within naïve CD4 and CD8 T-cell compartments with age. (A) The mean number of replicate TCRB sequences was used as an estimate of approximate clonal sizes. The frequency distribution of clonal size bins is shown as (log mean)  $\pm$  SD of the four young and the five elderly adults. Non-detectable clonal sizes were set at a frequency of 1 in  $10^7$ . (B) Replicate samples of naïve CD4 or naïve CD8 T cells were analyzed for the shared occurrence of TCRB sequences to estimate a clonality score, defined as the probability of two independently identified sequences originating from the same clone. Clonality scores were compared by Wilcoxon–Mann–Whitney test. The observed increase indicate inequality in clonal sizes in the elderly naïve T-cell repertoire with age with increasing number of large clones.

expected to have a lower richness. With advancing age, the number of memory T cells increases and end-differentiated effector CD8 T cells responsive to latent viruses, in particular CMV, accumulate (19, 20). In initial experiments, we compared richness in CD8 central, effector memory, and end-differentiated effector T cells in elderly individuals who did or did not have antibodies to CMV. Richness of effector cell populations was lower than that of central memory T cells and was further compromised in CMV-positive individuals (Fig. S2). To examine the effect of age independent of CMV-induced repertoire changes, we excluded individuals who had positive CMV serology and excluded terminally differentiated effector cells that are known to include clonally expanded effector T cells to latent viruses. Richness in the CD4 memory compartment was about 50-fold lower than in the naïve repertoire, with a lower bound of about 1 million different TCRB genes in each individual. Surprisingly, richness showed negligible contraction with age (Fig. 1 C and D). CD8 memory T cells were 5- to 10-fold less diverse than CD4 memory T cells, irrespective of age.

**Age-Dependent Clonal Expansion Within the Naïve T-Cell Compartment.** In addition to richness, distributions of clonal size could affect the functionality of the T-cell repertoire. Owing to limitations in sampling ( $5 \times 10^6$  naïve T cells out of a total repertoire of up to  $10^{12}$  cells per individual), estimates of clonal size distributions are only reliable for clones above a clonal size threshold. In the young as well as the old, most TCR sequences are derived from relatively small clones. In Fig. 2A, the frequencies of clones are plotted against clonal sizes (defined as the number of occurrences of identical sequences in the five combined samples of each subset). The frequency distributions for clones above the

detection threshold in the repertoires of young and elderly individuals clearly differed, with a small number of naïve T-cell clones showing a striking expansion with age. To compare the contributions of expanded clones to the repertoires, we used a “clonality score” summary metric that is independent of sequencing depth. This metric can be thought of as the probability that two sequence reads selected at random from different replicate library pools will be members of the same clone. Clonality scores in the elderly are approximately >100-fold higher for naïve CD8 T cells and >10-fold higher in CD4 T cells compared with younger individuals (Fig. 2B).

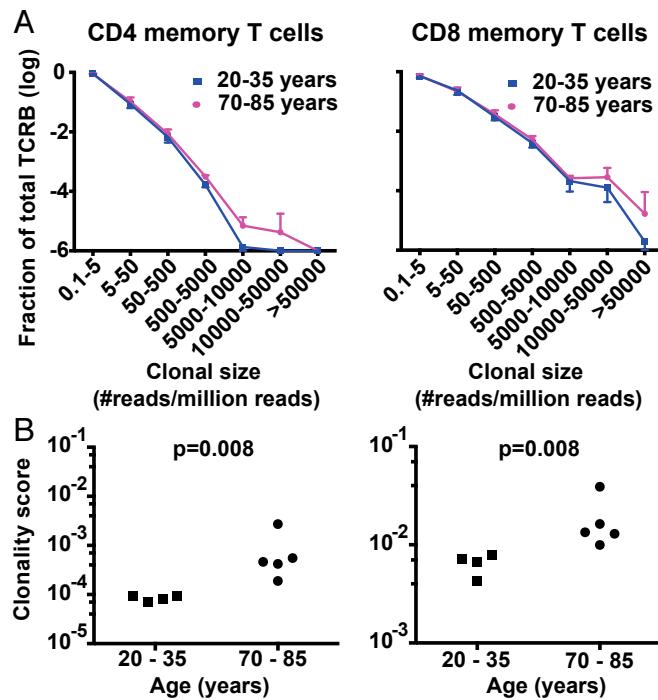
Recent studies have identified public TCR sequences that are shared by unrelated individuals (21, 22). Typically, public TCR sequences are those with simple junctions, such as those that lack N-region nontemplated nucleotides (22). To exclude that sequences detected more than once represented coincidentally identical TCR rearrangements rather than clonally expanded T cells, we analyzed sequences present at frequencies of greater than 0.01% in elderly individuals. A TCR sequence was called as public when found in more than one of the nine individuals studied. Twenty-three of the 483 naïve CD4 and 81 of the 878 naïve CD8 frequent TCRB sequences in old individuals were found in more than one individual (Table 1). In contrast, a higher number of 92 of the 483 most frequent CD4 ( $P < 0.001$ ) and 129 of 878 most frequent CD8 TCRB sequences ( $P < 0.05$ ) in the naïve repertoire of young individuals are shared in different individuals. The data indicate that the observed increased clonality in the elderly individuals represent true clonal expansions, whereas many of the apparently clonally expanded sequences in the young repertoires may reflect the presence of simple and public TCR rearrangements.

**Effect of Age on Clonality in the Memory T-Cell Compartment.** In contrast to the age-associated increases in clonality that we observed in naïve CD4 and CD8 cell compartments, there was little effect of age on the clonality of memory CD4 or CD8 T-cell populations. By virtue of memory T cells being present in larger clonal sizes than naïve T cells, clonal size distributions could be examined for a larger fraction of the repertoire. Distributions of clonal sizes were mostly identical for young and elderly individuals and an age-related increase in clonal sizes was seen only for the largest clones (Fig. 3A). Accordingly, clonality scores only slightly increased with age (Fig. 3B). Notably, CD8 exceeded CD4 memory T cells in clonal sizes.

**Clonally Expanded Naïve T Cells Express TCRB Sequences Distinct from Memory T Cells.** Effector T cells can revert to a phenotype sharing cell surface markers with naïve cells (23). To examine the possibility that the clonal expansions within the naïve compartment represented a contamination with effector T cells masquerading as naïve T cells, we analyzed all TCRB sequences that were found at more than 0.01% frequency in the naïve T-cell population for their representation in the memory population. The reverse analysis was done for clonally expanded sequences in the memory compartments. Results suggested that clonal expansions in the naïve compartment of elderly individuals were not contaminating memory cells. For example, of 121 TCRB sequences

**Table 1.** Increased clonality in the naïve T-cell compartment is not caused by an enrichment in public TCRB sequences

Age, y	CD4 naïve T-cell clones (n = 483)		CD8 naïve T-cell clones (n = 878)	
	Public	Unique	Public	Unique
20-35	92 (19%)	391 (80.9%)	129 (14.7%)	749 (85.3%)
70-85	23 (4.8%)	460 (95.2%)	81 (9.2%)	797 (90.8%)



**Fig. 3.** Greater clonality in the repertoire of CD8 than CD4 memory T cells. Frequency distributions of categorized clonal size bins (*A*) and clonality scores (*B*) for memory CD4 and CD8 T cells were determined as described in Fig. 2 for naïve T cells. Nondetectable clonal sizes were set at the minimal frequency of 1 in  $10^6$  (*A*). Clonality scores were compared by Wilcoxon-Mann-Whitney test. Large clonal expansions were more frequent in CD8 than in CD4 T cells with only minor influence of age on clonality in both compartments.

clonally expanded in the CD4 naïve T-cell compartment of one individual, 34 were unique for naïve T cells. Conversely, of 920 CD4 memory TCRB sequences, 449 were unique for memory cells. A similar distribution was seen for CD8 T cells: 215 of 379 clones originally identified in the CD8 naïve compartment were uniquely found in that compartment, whereas 349 of 846 memory sequences were not seen at all in naïve CD8 T cells. To further examine whether even the shared clonally expanded populations can be predominantly assigned to one compartment, the frequency of each of these shared sequences in each of the five naïve and five memory CD4 and CD8 T-cell replicate samples was determined. The heat plots in Fig. 4 show the frequency distributions of these clonally expanded CD4 and CD8 TCRB sequences for the different naïve and memory cell samples from two elderly adults. Each row represents one sequence and each lane an independent library of naïve or memory T cells. Although sequences were selected to be present in naïve and memory T cells, very few sequences were found at similar frequencies in both compartments. Importantly, TCRB sequences originally identified in the naïve compartment were virtually absent in memory cell pools, suggesting that these clonal expansions originated from naïve cells and maintained their naïve phenotype.

**Mechanisms of Clonal Expansion Within the Naïve T-Cell Compartment.** A likely interpretation of the increasing inequality of clonal sizes with age is that the homeostatic proliferation of naïve T cells is associated with fitness selection. Clonally expanded T cells might have a competitive growth advantage that in part could be conferred by increased responsiveness to homeostatic cytokines. To test this hypothesis, naïve CD8 T cells labeled with carboxy-fluorescein diacetate succinimidyl ester were cultured in the presence of IL-7 and IL-15. Cells that had undergone one or

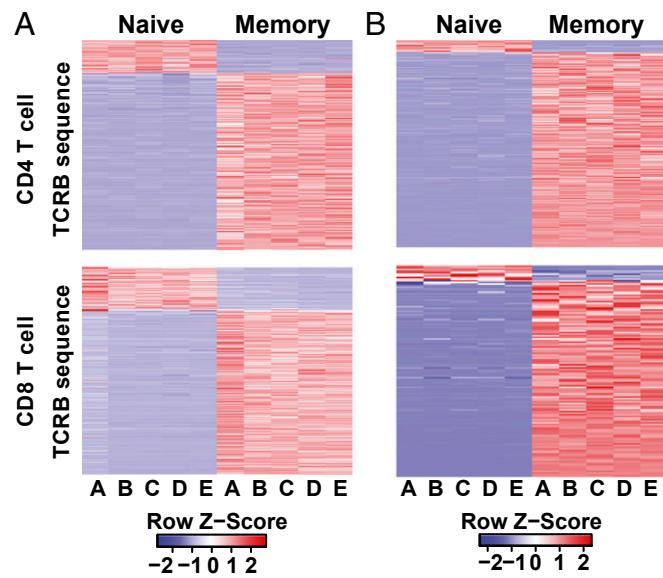
more or two or more divisions after 7 d were purified and sequenced. TCRB sequences from cultured T cells were analyzed for their presence in the replicate libraries of unstimulated naïve T cells. T cells that divided in culture in response to cytokine stimulation show an increased proportion of clones that were expanded in the peripheral blood (Fig. 5). The enrichment was more pronounced for T cells that divided more rapidly. In the population that had divided two or more times, large clones (defined as being found in four replicates of the original blood sample) are enriched compared with small clones (defined as present in one replicate) ( $P < 0.001$ ). In contrast, enrichment was not obvious when T cells purified for high expression of the IL-7 and IL-15 cytokine receptors CD127 and CD215, respectively, were analyzed.

## Discussion

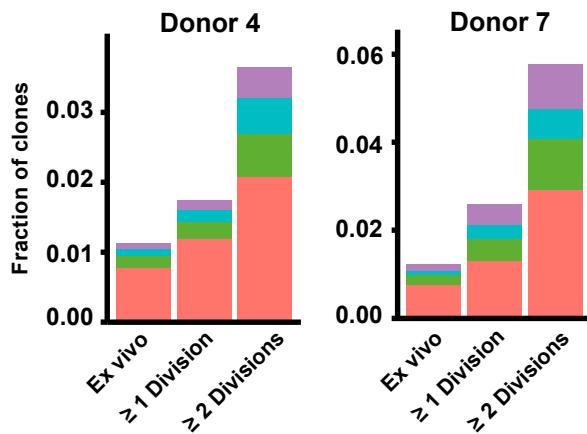
In this study we combined next-generation sequencing with a nonparametric statistical approach using the Chao2 estimator to estimate a lower bound for TCR richness. We found a higher richness in CD4 and CD8 naïve T cells than previous studies. Even though diversity contracts with age, we find that elderly individuals still possess a diverse T-cell repertoire. However, we observe robust clonal expansion with age in the naïve compartments, suggesting that homeostatic proliferation is associated with fitness selection. Finally, we found lower richness in CD8 than in CD4 memory cells, a difference that was preserved during aging.

Thymic involution is the most dramatic age-related change in the human immune system. Understanding whether the T-cell repertoire can be maintained in the absence of thymic activity and whether repertoire contraction contributes to the immune defects in the elderly is critical for designing possible interventions. Conclusions for the human repertoire from animal models are unreliable because the size of the T-cell compartment and mechanisms and kinetics of T-cell homeostasis are fundamentally different in humans and mice (3).

Whether thymic T-cell generation in humans is of any quantitative importance for the steady state of T-cell populations



**Fig. 4.** Characterization of clonally expanded naïve T cells. TCRB sequences that had frequencies of  $\geq 0.01\%$  and were found in the naïve as well as the memory compartment were analyzed for their frequencies in five independent naïve and memory replicate samples (samples A–E). Representative results of two elderly individuals (one in *A* and the other in *B*) are shown as heat maps using the color scheme from infrequent (blue) to frequent (red). The data indicate that T cells expanded in the naïve and memory compartments are different.



**Fig. 5.** Increased responsiveness of in vivo expanded naïve CD8 T cells to cytokine-induced proliferation. Naïve CD8 T cells were cultured with IL-7 and IL-15. TCRB sequences from naïve CD8 T cells that had divided equal to or more than once or twice were compared with sequences present in the peripheral blood repertoire of the individual. Results from two individuals are shown. The proportions of sequences from the cultured cells that were members of clones detected in four (purple), three (blue), two (green), or one (red) replicates of the original noncultured T-cell libraries from the peripheral blood are represented as cumulative bar graphs. The fastest-proliferating cultured T cells (right column) show enrichment of large clones found in four replicate libraries from the blood ( $P < 0.001$ ).

after the end of the growth period has been controversial. Some residual thymic tissue persists in elderly humans (24); however, even in the lymphopenic host after chemotherapy or bone marrow transplantation or in HIV patients after initiation of highly active antiretroviral therapy, resurgence of thymic activity does not occur in the majority of individuals older than 40–50 y (25). In the healthy adult, TCR excision circle (TREC) by-products of TCR rearrangement that do not replicate are the best surrogate marker for thymic activity. Mathematical modeling has suggested that the age-related decline in TREC is best explained by cell loss compensated by homeostatic naïve T-cell turnover rather than by declining thymic efflux. Thymic activity may not play a significant role in the maintenance of TCR diversity in adult life (26).

High-throughput DNA sequencing now enables extensive measurement of TCR populations, but estimation of TCR repertoire richness from sequencing of blood samples has remained a challenge. Here, we combined next-generation sequencing with a nonparametric statistical approach to estimate richness. The use of multiple replicate sequencing libraries generated from independent samples of T cells for each subset enabled us to identify the rare sequences that are critical for estimates of the number of unobserved species and overall TCR richness in the repertoire. We corrected for sequencing errors by excluding sequences that were related to detected peaks in sequence space. In contrast to previously used corrections, our approach does not eliminate essentially all rare clones, but it does still give a conservative appraisal of diversity.

Based on our estimate of 100 million TCRB gene rearrangements and considering that each TCR  $\beta$ -chain in the naïve T-cell repertoire combines with about 25 TCR  $\alpha$ -chains (11), the average clonal size of a naïve cell would be about 100–200 cells. This approximation is of the same magnitude as predicted from the dilution of TREC-bearing T cells in the naïve repertoire. In the neonate, naïve T cells proliferate to fill the compartment (27). Recent thymic emigrants in the newborn undergo three to four divisions in the periphery after TCRA gene rearrangement, thereby establishing a minimal clonal size of 10 cells (28). TREC frequencies decline further in subsequent years, suggesting that naïve T cells undergo approximately three more divisions during

the growth period in the presence of thymic activity, to give a final average naïve T-cell clonal size of about 100 cells.

Our analysis does not address directly whether a decline from about 20 million to 10 million TCR  $\beta$ -chain sequences leads to immune defects or holes in the repertoire in the elderly. Tetramer studies in noninfected individuals have suggested that the frequency of T cells specific for many exogenous viral peptides, or self-peptides, is on the order of 1 in 1 million (29), suggesting that the estimated elderly repertoires of more than 100 million TCR  $\alpha$ - $\beta$  dimers could be diverse enough to recognize most peptides bound to a given MHC molecule and avoid frank “holes” in the repertoire.

A striking age-related change in our studies was an increase in T-cell clonality in the naïve repertoire. The contribution of clonally expanded T cells to the observed repertoire increased by a factor of >100 for naïve CD8 and of >10 for naïve CD4 T cells compared with young adults. Analysis of these expanded clones showed that the clones in the naïve subsets were distinct from those in the memory compartments, although many of them were present in clonal sizes that were comparable to those of memory cells. If these naïve clones derive from uneven homeostatic proliferation, they maintain a naïve phenotype, in contrast to findings in mice, in which naïve T cells undergoing homeostatic proliferation tend to acquire a memory-like phenotype (30, 31).

Based on the limited number of cells that can be sampled in humans, our clonality index only allows conclusions on the impact of clones that are above a certain size. However, for naïve T cells age-associated increases were observed for the entire range of clonal sizes that could be assessed. This observation is consistent with the interpretation that homeostatic proliferation during aging is associated with increasing unevenness in clonal size distributions of the entire repertoire owing to peripheral selection. In support of this interpretation, we have recently performed an agent-based stochastic in silico simulation of repertoire complexity under homeostatic proliferation and have found only a minimal contraction in diversity over a human lifetime, as long as peripheral fitness selection during homeostatic proliferation can be ignored; even with no thymic activity after age 20 y and 90% shrinkage of the naïve T-cell compartment size (32). Depending on the initial clonal size, increasing variance in clonal abundances will not necessarily lead to clonal extinction. In our data, naïve CD8 T cells had a much higher clonality score than naïve CD4 T cells; however, richness in both compartments was very similar, suggesting a higher variance in clonal sizes in CD8 T cells (Figs. 1 and 2). A large number of homeostatic T-cell divisions or, as explored in our in silico simulation, a rather dramatic co-occurrence of selective forces is required before homeostatic proliferation leads to contraction in richness. However, the inequalities in clonal sizes with some large and many very small clones may generate a less complex T-cell response to peptide antigens, which may be more important than the fewer TCRs available in the repertoires of older compared with younger individuals. Such an unevenness cannot be improved by restoring thymic T-cell generation (32).

Age-associated skewing in clonal size distributions explains why some prior studies have observed a higher decline in diversity with aging. Because sequencing depths in most previous studies were not sufficient and/or the analytical design did not take into account increasing inequality in clonal size distributions, estimates of richness were not reliable. Based on this model, our previous report of a stable TCR repertoire up to the seventh decade of life with a sudden change thereafter indicates an occurrence of increasing T-cell clonal expansions late in life (33). This interpretation is also consistent with observations in a non-human primate model (34).

Homeostatic proliferation and survival of naïve T cells are thought to require the recognition of self-antigen in the presence

of homeostatic cytokines (30, 35). Inequalities in clonal sizes could therefore reflect a peripheral selection based on cytokine responsiveness and/or self-recognition. In support of this interpretation, *in vitro* culture of naïve T cells in the presence of homeostatic cytokines, but absence of foreign antigens, selected for TCR sequences clonally expanded *in vivo*. Homeostatic proliferation associated with a peripheral selection may therefore result in a more autoreactive repertoire (36). This may explain why lymphopenia-induced homeostatic proliferation confers an increased risk for autoimmunity (37, 38) and supports the model that increased homeostatic proliferation and associated changes in the repertoire found in patients with rheumatoid arthritis may predispose for the disease (39–41).

Surprisingly, and in contrast to naïve T cells, the impact of age on the memory T-cell repertoire was minimal. Although the size of the memory compartment is known to increase with age, richness did not change significantly, and clonality increased only for the very largest clones. This observation is particularly surprising for CD8 T cells where the terminally differentiated effector T-cell population tends to increase at the expense of central and effector memory T cells (17). It should be noted, however, that we have excluded individuals who were CMV-positive to analyze age effects without the confounding factor of CMV-induced repertoire changes.

Our data highlight striking differences in the repertoires of CD4 and CD8 T memory cells, independent of age. Richness was higher in CD4 memory than in CD8 T cells, whereas clonality was higher in CD8 memory T cells. The prominent clonality in CD8 memory T cells is consistent with previous spectratyping studies demonstrating clonal peaks in the CD8 memory repertoire (42). Our studies now show that the difference between

human CD4 and CD8 memory T cells is not limited to clonally expanded CD8 T cells, but also includes a globally decreased richness of the entire CD8 memory repertoire. Given that richnesses in naïve CD4 and CD8 T cells are approximately equal, our data suggest that formation or maintenance of the memory repertoire is more constrained for CD8 than for CD4 T cells. Future studies of the repertoires of antigen-specific T cells after vaccination or infection in humans will be required to further explore the consequences of T-cell subset-specific repertoire contractions and the contribution of clonal expansions to the increased vulnerability of the elderly to common pathogens.

## Materials and Methods

TCRB cDNA libraries were generated from five replicate samples of FACS-sorted naïve and memory CD4 and CD8 T cells from apheresis samples of young and elderly healthy adults and sequenced with an Illumina MiSeq sequencer. TCRB repertoire richness was determined by applying the Chao2 nonparametric estimator of the lower bound of species richness in a population after correcting for possible sequencing errors and eliminating TCRB sequences that are close to peaks in sequence space. A clonality score, adapted from the Gini-Simpson index, was determined using the lymphclon inference algorithm. A detailed description of the experimental design and procedures and the statistical analysis is given in *SI Materials and Methods*. Primers are described in **Table S3**.

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# Supporting Information

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## SI Materials and Methods

**Study Subjects.** Platelet donor apheresis lymphocytes were obtained from young (aged 20–35 y) and elderly (aged 70–85 y) adults from the Stanford Blood Center. All individuals were healthy, regular platelet donors without a history of autoimmune disease, diabetes mellitus, or chemotherapy and were analyzed for their CMV serology. Informed consent for research use of blood samples was provided by all participants.

**Cell Purification and RNA Extraction.** T cells were enriched by negative selection using the human T-cell RosetteSep enrichment kit (StemCell Technologies) and then stained with FITC-CD3- (BioLegend), V450-CD4- (Affymetrix eBioscience), PECy7-CD8-, PerCP/Cy5.5-CCR7- (BioLegend), PE-CD28- and APC-CD45RA- (BD Biosciences) antibodies as well as Live/Dead Fixable Aqua Dead cell stain dye (Life Technologies), followed by cell sorting using a BD Aria3 cell sorter to obtain naïve CD4 T cells ( $CD3^+CD4^+CCR7^-CD45RA^{high}CD28^+$ ), naïve CD8 T cells ( $CD3^+CD8^+CCR7^-CD45RA^{high}CD28^+$ ), memory CD4 T cells ( $CD3^+CD4^+CD45RA^-$ ), and memory CD8 T cells ( $CD3^+CD8^+CD45RA^-$ ). For young individuals, five replicates with  $1 \times 10^6$  cells per aliquot of naïve CD4, naïve CD8, and memory CD4 T cells and five replicates with  $0.25 \times 10^6$  per aliquot of memory CD8 T cells were collected. For naïve CD8 T cells in elderly individuals, five replicates of  $0.25 \times 10^6$  cells were collected; cell numbers for all other populations were the same as in young individuals. In additional experiments, central memory ( $CD3^+CD8^+CCR7^-CD45RA^-$ ), effector memory ( $CD3^+CD8^+CCR7^-CD45RA^-$ ), and terminal effector memory ( $CD3^+CD8^+CCR7^-CD45RA^{high}CD28^-$ ) CD8 T cells from CMV-positive and CMV-negative elderly individuals were sorted and five replicates with  $0.2 \times 10^6$  cells per aliquot of each CD8 T-cell population were collected. In two elderly individuals,  $CD4^+CD25^{high}CD127^{low}$  (Treg) and  $CD127^+$  and  $CD215^+$  naïve  $CCR7^+CD45RA^{high}CD28^+$  CD8 $^+$  were purified for analysis. RNA was extracted using AllPrep DNA/RNA mini kit (Qiagen). cDNA was generated using SuperScript III reverse transcription kit (Invitrogen) with random hexamer oligonucleotides.

**Cytokine-Mediated in Vitro T-Cell Expansion.** T cells were stained with V450-CD4-, PECy7-CD8-, APC-CD45RA-, and PerCP/Cy5.5-CCR7- antibodies, followed by cell sorting to obtain naïve CD8 T cells ( $CD3^+CD8^+CCR7^-CD45RA^{high}$ ). Cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured in vitro in the presence of 10 ng/mL IL-15 and 10 ng/mL IL-7 (Peprotech). After 7 d of culture, T cells were stained with V450-CD4, APC-CD3, and PECy7-CD8, followed by cell sorting to obtain T cells ( $CFSE^{low}CD8^+CD3^+$ ) that had divided in response to cytokine stimulation. RNA was extracted using RNA micro kit (Qiagen). cDNA was generated using SuperScript VILO MasterMix (Invitrogen).

**Generation of T-Cell Receptor Beta Gene Libraries.** To amplify rearranged T-cell receptor beta (TCRB) genes from cDNA, 39 forward primers, each specific to one or two functional TCRBV segments, and one reverse primer specific to the CB segments were designed based on the repertoire of TCR germ-line segments present in the ImMunoGeneTics (IMGT) database ([www.imgt.org](http://www.imgt.org)) (Table S3). A 12-bp nucleotide unique “barcode” sequence was inserted in the reverse primer between the CB sequence and Illumina universal reverse sequence. First PCR amplification was performed using a Multiplex PCR kit (Qiagen)

with 3  $\mu$ L of template cDNA, 50 pmol of CB primer, and 1.28 pmol of each V primer per 25  $\mu$ L of reaction for 15 min at 95 °C 15, followed by 30 cycles of 94 °C for 30 s, 59 °C for 90 s, and 72 °C for 60 s, then 72 °C for 10 min. Full-length Illumina sequencing adaptors were added in a second 15-cycle PCR with 0.5  $\mu$ L of the first PCR product and 10 pmol of forward and reverse Illumina adaptor primers. The final PCR products were pooled in equal amounts and separated on 2% agarose gel. PCR products of 350–450 bp were excised and extracted using a Qiaquick Gel Extraction Kit (Qiagen).

**Illumina Sequencing and Sequence Data Analysis.** TCRB libraries were sequenced with an Illumina MiSeq sequencer (2  $\times$  250-bp paired-end reads). Each paired-end read was first trimmed to 150 bp on each side and then assembled into a single sequences using COPE (1). The reads were then mapped to reference sequences in the IMGT information system ([www.imgt.org](http://www.imgt.org)) using modified IgBLAST. The TCRBV and TCRBJ regions for each sequence were annotated to best-matching sequences. CDR3 sequences were identified as nucleotide sequences between the second conserved cysteine at the 3' end of the VB gene segment and the conserved phenylalanine at the 5' portion of the JB segment. Sequences successfully mapped to known VB and JB segments and containing an in-frame CDR3 sequence and JB segment were accepted for further analysis.

**TCRB Repertoire Richness Statistics.** We used multiple replicate libraries generated from distinct aliquots of T cells for each subset and applied the Chao2 nonparametric estimator of the lower bound of species richness in a population (2, 3). The Chao2 approach is a standard tool in ecology and intends to make use of the information provided by all species in a dataset, rare or not. Instead of excluding all singleton sequences, as was done in previous studies, we corrected for sequencing errors by examining each putative clone  $S$  that appeared only in one replicate and decided whether it could be a sequencing error with respect to another clone using the following rule set:  $S$  is considered a sequencing error if another clone in the data from that T-cell subset (i) shares the same V and J annotations, (ii) has the same CDR3 length, (iii) has a Hamming distance of two or one to  $S$  in the CDR3 nucleotides, and (iv) has higher abundance. If we find another clone satisfying all four criteria, then the putative clone  $S$  is rejected as a clone.

Computing Chao2 entails examining the replicates for clones that are found in multiple replicates and contrasting this quantity to the number of clones found in only one in a mathematically informed manner. Chao2 essentially contrasts these two quantities to estimate the extent to which we have covered the full repertoire and uses that to inform how many species there are in total.

We used two strategies to estimate confidence intervals of the Chao2 estimator. First, we have produced an empirical confidence interval based on bootstrapping. We have used the BC<sub>a</sub> bootstrap from DiCiccio and Efron (4) that is a variant of bootstrapping designed in part for confidence intervals when the underlying bootstrap distribution is not symmetric about its center. Following standard technique, we converted the replicated data into sufficient statistics necessary to compute the Chao2, in particular, the per-clone incidence frequencies across the replicates (i.e., the number of replicates that contain the clone). We performed sampling with replacement on this sufficient statistic over all clones. In our bootstrap estimates, we performed

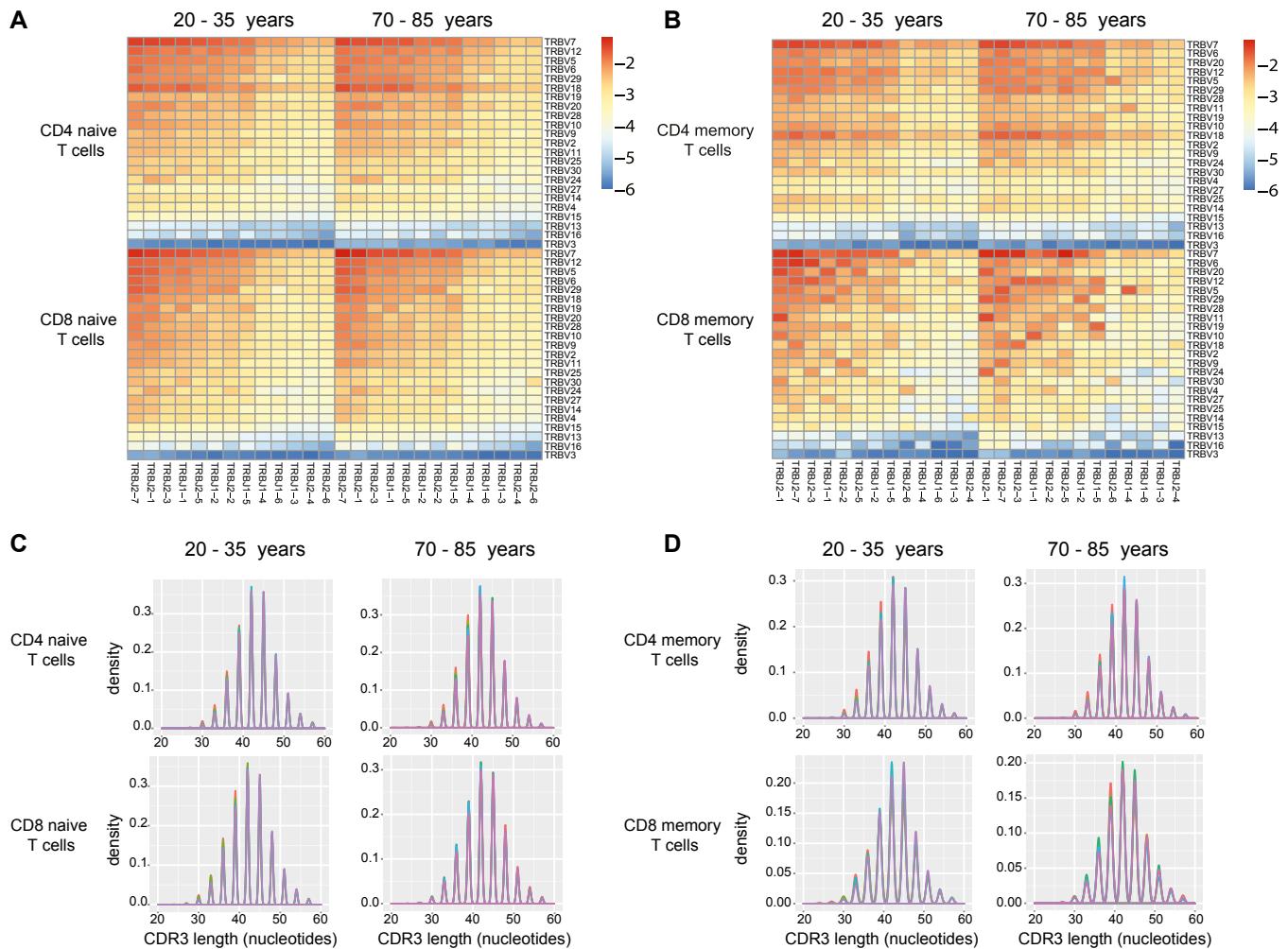
1,000 bootstrapping iterations for each estimate. Second, we estimated the confidence intervals using the formula developed by Chao (2).

Fewer cells were available for analysis in our CD8 memory T-cell samples compared with the CD4 memory cells (5 replicates of 250,000 each vs. 5 replicates of 1,000,000 each); therefore, we ran simulations to evaluate the robustness of our estimates of richness, given the differences in sampling. We generated an underlying repertoire with an uneven Zipf distribution (power = -0.5), spread across 1 million clones. From this repertoire, we sampled 5 replicate libraries, for 250,000 virtual cells per replicate, equivalent to the number of CD8 memory cells studied in our experiments. To simulate PCR amplification, each virtual cell was subject to a standard lognormal scaling. Across each of the 100 random iterations of these noisy samplings, we calculated a Chao2 repertoire estimate of richness. The resulting estimates of a mean of 924,400 with a SD of 1756 were very close to the true value of 1 million different TCRs. For comparison, we also

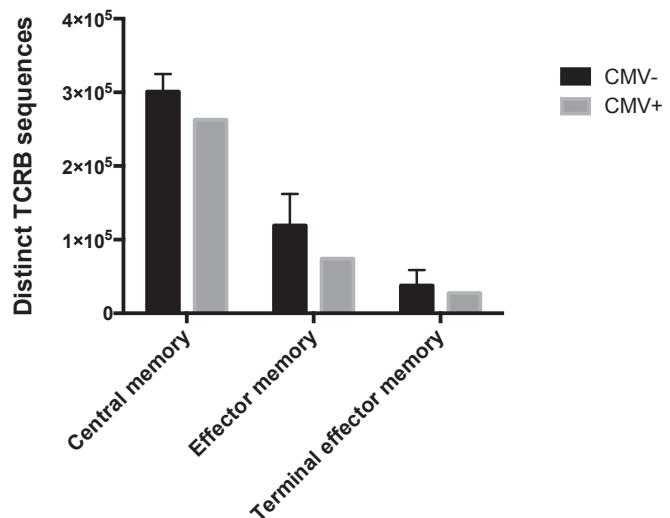
performed simulations using sampling depths equivalent to those obtained for CD4 memory T-cell experiments (5 replicates of 1 million cells each). This resulted in a mean of 945,800 and a SD of 800, demonstrating the robustness of the approach to variation in sample sizes.

**Clonality Statistics.** The “clonality score” was recently developed as a measure to estimate contribution of clonally expanded sequences within a repertoire, as an adaptation of the Gini–Simpson index modified for application to multiple replicate sequencing datasets and corrected for covariance between replicate sequence libraries (5, 6). To perform inference of the clonality score, we used the lymphclon package available on R CRAN to perform accurate estimation of this quantity on the repertoires. The lymphclon inference algorithm explicitly takes advantage of multiple replicated sequencing experiments by modeling the experimental covariances and the per-replicate amplification differences. A description of this methodology is available in ref. 7.

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**Fig. S1.** Influence of age on the repertoire of rearranged TCRB sequences. (A and B) Naïve (A) and memory (B) CD4 and CD8 T cells were purified from young and elderly individuals and transcribed TCRB genes were sequenced. Mean frequencies of TCRB V-J combinations are illustrated as heat plots. The order of the TCRBV gene segments on the horizontal and the TCRBJ gene segments on the vertical axis is maintained for all subsets for easy visual comparison. (C and D) TCRB CDR3 lengths were determined for all sequences within each T-cell subset and the frequencies of their occurrences were determined. Results are shown as density blots; colored lines indicate the different young or elderly individuals and are largely overlapping.



**Fig. S2.** Comparison of TCRB repertoire richness in memory CD8 T-cell subsets. Replicate samples of CD8 memory subset TCRB libraries were analyzed as described in Fig. 1. Estimates of lower boundaries in TCRB nucleotide richness are shown for two CMV-negative and one CMV-positive elderly individual.

**Table S1.** Number of TCRB sequence reads obtained for individual replicates

Donor	Age	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5
Naïve CD4 T cells						
1	74	459,108	490,801	519,560	513,972	537,717
2	35	358,400	326,179	377,882	372,326	380,736
3	30	696,485	820,273	844,549	851,532	950,305
4	76	388,345	433,703	461,075	449,440	397,094
5	83	170,862	267,261	304,165	311,096	327,667
6	33	580,865	464,626	592,259	581,854	559,111
7	75	356,701	376,339	395,280	402,975	402,998
8	24	329,853	352,379	343,058	351,383	372,452
9	79	335,484	336,665	344,163	354,635	339,293
Naïve CD8 T cells						
1	74	659,178	437,717	445,332	430,420	450,413
2	35	384,930	351,888	464,414	407,943	401,188
3	30	395,573	326,661	359,539	374,804	337,547
4	76	629,667	667,288	692,752	665,063	694,879
5	83	261,444	322,898	297,672	453,983	714,078
6	33	414,307	411,069	395,779	430,989	447,877
7	75	322,937	459,150	516,841	474,051	428,739
8	24	378,205	316,456	365,923	343,686	329,259
9	79	336,438	327,133	359,927	360,780	332,226
Memory CD4 T cells						
1	74	312,382	320,624	320,153	373,334	393,616
2	35	373,895	476,292	398,892	397,240	479,418
3	30	465,797	457,311	412,081	390,651	422,076
4	76	352,640	354,381	351,808	385,821	410,303
5	83	132,458	194,698	203,693	180,100	174,267
6	33	161,682	219,430	191,816	200,767	151,423
7	75	409,548	347,105	361,875	338,796	380,941
8	24	265,117	239,266	258,125	241,409	269,853
9	79	295,194	307,580	292,062	300,259	266,490
Memory CD8 T cells						
1	74	299,719	346,161	515,793	445,017	320,588
2	35	228,005	287,019	208,819	211,622	210,139
3	30	428,693	428,517	395,586	462,686	414,090
4	76	431,991	354,757	377,418	336,192	332,785
5	83	218,178	243,554	277,216	209,957	229,757
6	33	159,346	132,763	102,696	161,929	164,516
7	75	244,633	179,847	196,398	221,402	185,952
8	24	335,145	278,849	311,536	282,872	205,952
9	79	276,389	298,710	317,016	299,887	306,782

**Table S2.** Confidence intervals of estimated lower boundaries in TCRB richness

Donor ID	T-cell compartment	Chao2 estimate	95% BC <sub>a</sub> confidence interval		95% confidence interval (Chao2 log-normal method)	
			Lower	Upper	Lower	Upper
1	CD4 naïve	7.93E+06	7.86E+06	8.00E+06	7.86E+06	8.00E+06
1	CD8 naïve	1.15E+07	5.77E+05	5.77E+05	1.13E+07	1.17E+07
1	CD4 memory	1.09E+06	1.08E+06	1.10E+06	1.08E+06	1.10E+06
1	CD8 memory	1.84E+05	1.81E+05	1.87E+05	1.81E+05	1.87E+05
2	CD4 naïve	8.11E+07	7.85E+07	8.39E+07	7.84E+07	8.40E+07
2	CD8 naïve	7.74E+07	7.53E+07	7.97E+07	7.54E+07	7.95E+07
2	CD4 memory	2.17E+06	2.15E+06	2.19E+06	2.15E+06	2.19E+06
2	CD8 memory	2.10E+05	2.06E+05	2.14E+05	2.06E+05	2.15E+05
3	CD4 naïve	6.91E+07	6.78E+07	7.06E+07	6.77E+07	7.06E+07
3	CD8 naïve	6.46E+07	6.29E+07	6.63E+07	6.29E+07	6.63E+07
3	CD4 memory	1.67E+06	1.65E+06	1.68E+06	1.65E+06	1.68E+06
3	CD8 memory	3.70E+05	3.65E+05	3.76E+05	3.64E+05	3.76E+05
4	CD4 naïve	2.73E+07	2.69E+07	2.78E+07	2.69E+07	2.78E+07
4	CD8 naïve	5.76E+07	5.57E+07	5.96E+07	5.57E+07	5.96E+07
4	CD4 memory	1.12E+06	1.10E+06	1.13E+06	1.10E+06	1.13E+06
4	CD8 memory	2.24E+05	2.20E+05	2.27E+05	2.20E+05	2.28E+05
5	CD4 naïve	9.85E+06	9.69E+06	1.00E+07	9.69E+06	1.00E+07
5	CD8 naïve	1.02E+07	9.97E+06	1.04E+07	9.96E+06	1.04E+07
5	CD4 memory	6.77E+05	6.70E+05	6.85E+05	6.70E+05	6.85E+05
5	CD8 memory	1.41E+05	1.39E+05	1.44E+05	1.39E+05	1.44E+05
6	CD4 naïve	1.23E+08	8.93E+05	8.93E+05	1.18E+08	1.28E+08
6	CD8 naïve	9.04E+07	8.80E+07	9.31E+07	8.80E+07	9.29E+07
6	CD4 memory	1.03E+06	1.02E+06	1.04E+06	1.02E+06	1.04E+06
6	CD8 memory	1.80E+05	1.76E+05	1.84E+05	1.75E+05	1.84E+05
7	CD4 naïve	2.72E+07	2.66E+07	2.77E+07	2.67E+07	2.77E+07
7	CD8 naïve	4.27E+07	4.09E+07	4.45E+07	4.10E+07	4.45E+07
7	CD4 memory	1.35E+06	1.34E+06	1.37E+06	1.34E+06	1.37E+06
7	CD8 memory	1.22E+05	1.20E+05	1.25E+05	1.19E+05	1.25E+05
8	CD4 naïve	9.34E+07	9.06E+07	9.65E+07	9.06E+07	9.63E+07
8	CD8 naïve	7.62E+07	7.41E+07	7.85E+07	7.40E+07	7.85E+07
8	CD4 memory	1.52E+06	1.51E+06	1.54E+06	1.51E+06	1.54E+06
8	CD8 memory	4.17E+05	4.10E+05	4.24E+05	4.10E+05	4.24E+05
9	CD4 naïve	6.61E+06	6.55E+06	6.67E+06	6.54E+06	6.67E+06
9	CD8 naïve	1.07E+07	1.05E+07	1.09E+07	1.05E+07	1.09E+07
9	CD4 memory	8.65E+05	8.57E+05	8.72E+05	8.58E+05	8.73E+05
9	CD8 memory	1.52E+05	1.50E+05	1.55E+05	1.49E+05	1.55E+05

**Table S3.** TCRB-specific PCR primer sequences

TRBV10-1-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAAAGTCAGATGGCTACAGTGTCTCTAG
TRBV10-2-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtATCCAGATCCAAGACAGAGAATTCCCC
TRBV10-3-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAAAGTCAGATGGCTATAAGTGTCTCTAG
TRBV12-5-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAAAGTCAGATGGCTATAAGTGTCTCTAG
TRBV13-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAAAGTCAGATGGCTATAAGTGTCTCTAG
TRBV14-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAAAGTCAGATGGCTATAAGTGTCTCTAG
TRBV16-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAAAGTCAGATGGCTATAAGTGTCTCTAG
TRBV18-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAAAGTCAGATGGCTATAAGTGTCTCTAG
TRBV19-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAAAGTCAGATGGCTATAAGTGTCTCTAG
TRBV2-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAAAGTCAGATGGCTATAAGTGTCTCTAG
TRBV20-1-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAAAGTCAGATGGCTATAAGTGTCTCTAG
TRBV24-1-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAAAGTCAGATGGCTATAAGTGTCTCTAG
TRBV25-1-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAAAGTCAGATGGCTATAAGTGTCTCTAG
TRBV27-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAAAGTCAGATGGCTATAAGTGTCTCTAG
TRBV28-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAAAGTCAGATGGCTATAAGTGTCTCTAG
TRBV6-1-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAAATGTCCTCAGATTAAACAAACGGGAGTT
TRBV6-3-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAACTCTGATGGCTACAATGTCCTCAG
TRBV6-4-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAACTAGTGTCTCAGAGCAAACAGATG
TRBV6-8-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAACTAGTGTCTCAGAGCAAACAGATG
TRBV6-9-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAACTAGTGTCTCAGAGCAAACAGAGG
TRBV7-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAACTAGTGTCTCAGAGCAAACAGAGG
TRBV9-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAACTCTGAAACGATTCTCCGACAACAG
TRVB11-1.11-2.11-3-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAACTTGCTTAAGGATCGATTCTGC
TRVB12-3.12-4-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAGGGATGCCGAGGATCGATTC
TRVB15-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAACTCCGATCTCCAATCCAGGAG
TRVB29-1-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAACTCCGATCTCCAACATTC
TRVB3-1-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAACTCCGATCTCCAACATTC
TRVB30-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAACTCCGATCTCCAACATTC
TRVB4-1.4-2.4-3-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAACTCCGATCTCCAACATTC
TRVB5-1-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAACTCCGATCTCCAACATTC
TRVB5-4-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAACTCCGATCTCCAACATTC
TRVB5-5-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAACTCCGATCTCCAACATTC
TRVB5-6-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAACTCCGATCTCCAACATTC
TRVB5-8-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAACTCCGATCTCCAACATTC
TRVB6-5.6-6-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAACTCCGATCTCCAACATTC
TRVB7-2-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAGGGCTGCCAGTGATCG
TRVB7-6.7-7-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAGGGCTGCCAGTGATCG
TRVB7-8-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAGGGCTGCCAGTGATCG
TRVB7-9-F	GGCATTCCTGCTGAACCGCTTCCGATCTNNNNactaggtAGGGCTGCCAGTGATCG
TCRBconst	ACACTTTCCCTACACGACGCTTCCGATCTNNNNactcgtaCGACCTGGGTGGAACAC

Lowercase nucleotides represent barcode sequences.