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Longterm maintenance of human naïve T cells through *in situ* homeostasis in lymphoid tissue sites

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Abstract

Naïve T cells develop in the thymus and coordinate immune responses to new antigens; however, mechanisms for their long-term persistence over the human lifespan remain undefined. Here, we investigated human naïve T cell development and maintenance in primary and secondary lymphoid tissues obtained from individual organ donors aged 3 months-73 years. In the thymus, the frequency of double-positive thymocytes declined sharply in donors over age 40 coincident with reduced recent thymic emigrants (RTE) in lymphoid tissues, while naïve T cells were functionally maintained predominantly in lymph nodes (LN). Analysis of TCR clonal distribution by CDR3 sequencing of naïve CD4⁺ and CD8⁺ T cells in spleen and LNs reveal site-specific clonal expansions of naïve T cells from individuals >40 years of age with minimal clonal overlap between lymphoid tissues. We also identified biased naïve T cell clonal distribution within specific lymph nodes based on VJ usage. Together these results suggest prolonged maintenance of naïve T cells through *in situ* homeostasis and retention in lymphoid tissue.

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Data and Materials Availability: The flow cytometry data for this study will be deposited in the ImmPort database (<https://immunport.niaid.nih.gov>) and the TCR sequencing data are available through the Adaptive Biotech website <https://clients.adaptivebiotech.com/publishedProjects>.

Introduction

The ability to respond to new antigens is mediated largely by naïve T cells, which are generated in the thymus and emerge into the periphery by migrating through blood and lymphatics. The production of new naïve T cells from the thymus is highest at birth and during infancy, and there is an established reduction in thymic function and volume beginning in puberty (1). It is not understood how and whether human naïve T cells are maintained in the context of decreasing thymic output throughout a lifetime. Moreover, human lifespan continues to increase, and the ability of individuals to maintain health and be free of infectious/chronic diseases even in advanced years (2, 3) suggests that the human immune system has specific mechanisms in place for maintaining functionality over many decades. However, identifying mechanisms for preserving immunity in humans remains difficult to assess and investigate.

The capacity of T cells to recognize diverse antigens depends on their TCR specificity. TCR gene rearrangement in developing thymocytes results in each new naïve T cell expressing a unique TCR, which in humans can comprise over 100 million different specificities (4). When activated by antigen/MHC, clones of naïve T cells proliferate and differentiate to activated/effector T cells, of which a proportion can persist as memory T cells. While naïve T cells predominate in peripheral blood at birth, there is a gradual accumulation of memory T cells with age, and naïve T cells comprise, on average, 20-40% of circulating CD4⁺ or CD8⁺ T cells in adults (5-7). In mice, maintenance of naïve T cells is largely dependent on thymic output, while in humans, naïve T cell maintenance in blood appears driven by peripheral, homeostatic expansion (8), which could occur via tonic signaling or homeostatic cytokines such as IL-7 (9). It is not known whether these apparent distinctions in naïve T cell maintenance between mice and humans are due to the sampling site (spleen and LN in mice compared to blood in humans), lifespan differences (1-2 years in mice versus >80 years in humans), or other factors. In humans, blood is the major accessible sample, yet only contains 2-3% of the total T cell complement (10), while naïve T cells are generated in the thymus, seeded into and become activated in secondary lymphoid organs.

We have set up a resource to obtain multiple tissues from human organ donors through a collaboration and research protocol with the organ procurement organization for the New York metropolitan area (LiveOnNY). This unprecedented access to human tissues has enabled study of human T cell subsets, function, and clonal organization in lymphoid and mucosal tissues from diverse individuals of all ages (7, 11, 12). From collective analysis of over 70 donors, naïve T cells were found to persist in frequencies of 20-40% predominantly in lymph nodes, spleen and blood in young adults into the seventh decade of life (7, 11, 12). We hypothesized that these lymphoid sites could serve as reservoirs for longterm maintenance of naïve T cells, and their characterization could reveal mechanisms that cannot be elucidated from studies in blood. We further considered whether specific clones of naïve T cells exhibited compartmentalization as described for subsets of memory T cells (12, 13).

Here, we present a detailed analysis of human naïve T cell development and maintenance in primary and secondary lymphoid tissues obtained from individual organ donors, aged 2 months to 73 years of age. We dissected mechanisms for naïve T cell maintenance through

analysis of T cell phenotype and TCR clonal distribution by CDR3 sequencing of naïve CD4⁺ and CD8⁺ T cells in spleen and LNs from donors aged 1-60 years. Our results reveal that each lymphoid tissue site contains a unique complement of naïve T cell clones with minimal overlap between tissues, that clonal expansions of naïve T cells are observed, particularly in individuals >40 years, and these expansions likewise are contained within specific sites. Together, these results demonstrate localization-dependent mechanisms for the maintenance of human naïve T cells through *in situ* homeostasis, with potential effects on priming and localization of immune responses later in life.

Results

Structural alterations and reduced DP thymocytes after age 40

We obtained thymus tissues from donors of different ages and examined thymus integrity by histological analysis and thymocyte composition by flow cytometry. Thymic tissue undergoes age related atrophy evidenced by a decrease in thymic epithelial space with organ volume further replaced by fatty tissue (14, 15). To ensure that we were isolating T cell populations from viable thymus and not fat, we performed H and E staining of isolated tissues and looked for evidence of Hassall Corpuscles (HC) that are a structural hallmark of the human thymus and consist of epithelial cells within the thymic medulla (16). Thymic tissues obtained from pediatric and adult donors of different ages had visible HC structures, with pediatric thymus tissue exhibiting a significantly higher density of HC of smaller size compared to adult thymii with larger HC structures (Fig. 1A, B), demonstrating age-associated structural changes within active thymic tissue in adults.

Thymocytes isolated from active thymic tissue exhibited the canonical thymocyte subpopulations delineated by CD4 and CD8 expression into subsets with characteristic frequencies including double positive (DP) CD4⁺CD8⁺ and single positive (SP) CD4⁺CD8⁻ (20%) or CD8⁺CD4⁻ (10%) cells with DP cells comprising the majority (60-80%) of total thymocytes (15). We found characteristic frequencies of DP and SP thymocytes in the thymus tissue of young donors (Fig. 1C, top and see Fig. S1 for gating strategy) and these subsets also exhibited characteristic CD69 staining of thymocyte subsets (17, 18) with DP exhibiting lower CD69 expression compared to SP subsets (Fig. 1C, lower). Interestingly, the frequency of DP populations was consistently 60-80% of thymocytes from infancy up until the fourth decade of life, and after age 40-50 years there was a steep decline in the percentage of DP cells to <15% of thymocytes (Fig. 1D). These findings show that active thymopoiesis does not exhibit a gradual decline but may cease abruptly at some discrete point in time after 40yrs of age.

Naïve T cells are differentially maintained based on lineage and lymphoid tissue site

We investigated the maintenance of naïve-phenotype T cells (CD3⁺CD45RA⁺CCR7⁺ CD4⁺ or CD8⁺ cells, see gating strategy in Fig. S1) in circulation, lymphoid, and mucosal tissues as a function of different age groups associated with high or low levels of thymopoiesis based on the results in Fig. 1. We stratified the age groups into pediatric donors (2 months-5 years), young adult donors (15-39 years) with active thymic output, and middle aged/older adult donors (>40 years, with the majority of donors between 40-60 years of age) with

diminished thymic output. In pediatric donors, appreciable proportions of naïve T cells could be found in all sites examined with the lowest proportions seen in small intestine and lungs as previously described (11). In young and older adults, the proportion of naïve T cells in mucosal tissues was negligible; however, substantial proportions were found in blood, spleen and LN (Fig. 2A). Between young and older adult age groups, the proportion of naïve T cells decreased significantly in spleen, ILN and LLN for both CD4⁺ and CD8⁺ T cells, and in blood for CD8⁺ T cells, while frequencies of naïve T cells in MLN was similar in all adult age groups for both CD4⁺ and CD8⁺ T cells (Fig. 2A, compare dashed to solid black lines, Table S2), indicating differential maintenance in various sites. Notably, even in the older adult ages, up to 20% of CD4⁺ T cells and 30-40% CD8⁺ T cells were maintained as naïve phenotype cells in lymph nodes, suggesting long-term maintenance of naïve T cells without continuous export from the thymus.

We further investigated the dynamics of naïve T cell loss and maintenance in specific lymphoid sites (spleen, ILN, LLN and MLN) for both CD4⁺ and CD8⁺ T cells and their dynamics within individuals (Fig. 2B,C). Overall, there was a higher proportion of naïve CD8⁺ T cells maintained in lymph nodes examined at all ages compared to naïve CD4⁺ T cells (Fig. 2B), with steep reductions in naïve T cell frequency in ILN, LLN and MLN by age 30-40 for CD4⁺ T cells, while frequencies for CD8 T cells were maintained in these sites in certain individuals until >50 years of age (Fig. 2C). In contrast to the differential maintenance of naïve CD8⁺ compared to CD4⁺ T cells in LN, the spleen exhibited a similar steep decline of naïve T cells beginning in the young adult years (early 20's) for both CD4⁺ and CD8⁺ T cells (Fig. 2A, B), which could be attributed to higher turnover of naïve T cells in spleen versus LN based on analysis of Ki67 in a limited number of donors (Fig. S2). Together, this analysis reveals previously unknown disparities in maintenance of naïve T cells over life in different lymphoid compartments, with certain lymph nodes serving as potential niches for long-term persistence of naïve phenotype cells, and spleen representing a transient compartment for naïve T cells during youth.

Loss of RTE content and compartmentalization in lymphoid tissues over 40 years of age

Recent thymic emigrants (RTE) can be detected in the periphery by the presence of extra-chromosomal DNA resulting from the TCR- δ gene rearrangement event, which occurred during thymic development (19-21). These T cell receptor excision circles (TREC) are present at measurable levels in RTE but gradually become diluted out with each cell division due to homeostasis or antigen-driven activation (21). We sorted naïve (CD45RA⁺CCR7⁺) CD4⁺ and CD8⁺ T cells from lymphoid sites (spleen, ILN, LLN, MLN) and SP CD4⁺ or CD8⁺ thymocytes and assessed TREC content using a well-established PCR assay (21, 22) (see methods) (Fig. 3A).

Overall the average TREC levels at a given age were similar between naïve CD4⁺ and CD8⁺ T cells. However, two types of age-associated changes in TREC levels were observed. First, there was an overall decrease in TREC content of naïve CD4⁺ and CD8⁺ T cells and thymic SP cells with age, with the most striking reduction in TREC levels to <1000 after 40 years of age (Fig. 3A and see Table S3 for individualized TREC values), providing additional evidence for an abrupt reduction in thymic output in humans after age 40.

The second age-associated change in TREC levels was observed between tissues within an individual donor. In pediatric and young adult lymphoid tissues, the TREC content of naïve T cells was not equivalent within a single individual (Fig. 3A; different tissue values for each individual share the same color, and Table S3). For example, in a 3yr old donor (Donor 82, Table S3), the highest TREC content of naïve CD4⁺ T cells was found in the thymus followed by ILN and then spleen, whereas for a 12 year old donor, higher TREC levels were detected in splenic naïve T cells, followed by LLN and ILN (Fig. 3A and Table S3). Dissimilar TREC levels between naïve T cells isolated from different sites were observed mostly in individuals younger than 40 years of age (Fig. 3 and Fig. S3), with individuals over age 40 having comparable low TREC levels in distinct sites. Variations in TREC content between lymphoid sites of an individual could reflect differential thymic seeding and/or differences in activation or maintenance of naïve T cells *in situ*.

We also compared CD31 expression by naïve CD4⁺ T cells in donors of different ages (Fig. S4), as a marker of RTE (23). While the overall frequency of CD31 on total CD4⁺ T cells was indicative of reduced thymic output and seeding of naïve T cells in mucosal sites in adults (Fig. S4A,B), its expression by naïve-phenotype cells in adults did not decrease coincident with the decreased TREC levels found in donors (Fig. S4C). These results suggest that CD31 is not a good marker to gauge thymic output in adults.

Naïve T cell subsets maintain functionality with age

To assess whether naïve T cells in lymphoid tissue maintained their functionality and naiveté at different ages associated with presence or absence of thymic output, we measured the cytokine profile of naïve CD4⁺ and CD8⁺ T cells isolated from lymphoid tissues following anti-CD3/anti-CD28-mediated activation *ex vivo*. We assessed the production of multiple cytokines in culture supernatants to determine whether naïve T cells predominantly produced IL-2 or had acquired the capacity to produce effector cytokines more associated with memory T cell responses including IFN- γ . However, naïve T cells from all tissue sites (spleen, ILN and LLN) and from donors of diverse ages produced predominantly IL-2 with low-to-negligible levels of IFN- γ (Fig. 3B and Table S4), IL-4 and IL-10 (Fig. S5). Memory T cells, by contrast, exhibited an enhanced capacity to produce substantial levels of IFN- γ , IL-4 and IL-10 compared to naïve T cells within the same site from the same donor (Fig. 3C, Fig. S5 and Table S4). Together, these results indicate that naïve T cells defined by phenotypic expression of CD45RA and CCR7 remain functionally naïve even in the presence of waning thymic output.

TCR sequencing of T cell populations shows differences in sequence diversity both with age and subset

The above analyses revealed that lymph nodes served as potential reservoirs for functional maintenance of naïve T cells and that naïve CD4⁺ and CD8⁺ T cells were differentially maintained as a function of age. To gain new insights into the mechanisms for human naïve T cell maintenance in these key sites during periods of active (<40 years of age) and low or negligible (>40 years) thymic output, we analyzed the clonal distribution of naïve T cells within and between tissue sites using the ImmunoSEQ platform to sequence all possible human TCR CDR3 β sequences (24, 25). ImmunoSEQ has been used to dissect the clonal

origin of memory subsets (26), and for detecting antigen-specific T cell clones in clinical samples (27, 28). Here, we applied ImmunoSEQ to assess how human naïve T cells were clonally distributed in spleen (SP), inguinal lymph node (ILN) and lung-draining lymph node (LLN). We extracted DNA samples from CD4⁺ and CD8⁺ T cells from a total of 19 donors, including naïve subsets from 13 donors and effector-memory (TEM) populations from 10 donors, of which naïve and TEM subsets were analyzed together in tissues from 4 of these donors (Table S5). From these data, we assessed naïve TCR diversity in different tissues and donors as a function of age, and the tissue overlap of individual naïve T cell clones regarding the extent to which a clonal population in one tissue is found in other sites from the same individual. We compared these aspects of TCR clonal diversity and distribution in the corresponding TEM subset as our previous studies had revealed differences in diversity and tissue overlap in CD4⁺ and CD8⁺ TEM cells, although age was not a contributing factor (7).

For all T cell subsets used for TCR analysis, we obtained read numbers of adequate size for calculating diversity and tissue overlap (10^5 - 10^6 reads/sample, see Table S5). We defined a distinct clonotype by nucleotide sequence and assessed the clonal diversity of naïve T cells in tissues as a function of age using the Simpson's index, a diversity measure which gives the average probability that two clonotypes randomly selected from a population are identical (see methods). Analysis of naïve TCR repertoires from spleen, ILN, and LLN from 11 donors aged 1-60 allowed us to assess the overall influence on diversity due to decreasing thymic output. We identified an overall drop in diversity (increased Simpson's index) after 40 years of age, though with high variability across donors (Fig 4A). For naïve CD8⁺ T cells, there was a broader range of values between samples of a given age range, yet higher diversity was still observed at ages >40 years (Fig. 4A, right). We did not find significant differences in naïve T cell diversity and/or clonal expansion between lymphoid sites when comparing spleen, ILN and LLN from all donors examined (Fig. 4A and Fig. S6).

We further assessed how expansion of naïve clones compared to antigen-experienced TEM clones. Since the Simpson's index assigns greater importance to clones present at a higher frequency, we posited that the frequency of the largest clone in each T cell population would be sufficient to distinguish differences in the diversity of these two subtypes (Fig. 4B). Among the TEM repertoire, the highest frequency clones were significantly greater than those in the naïve repertoire, with the most expanded TEM clones present in frequencies 50 to 200-fold greater than the maximally expanded naïve T cell clones (Fig. 4B). Moreover, naïve and TEM repertoires for CD8⁺ T cells were more expanded than the corresponding CD4⁺ T cell subsets (Fig. 4B).

TCR diversity of Naïve and TEM cells was compared on the level of VJ cassette recombination. Clone frequencies were calculated for all samples, and samples of the same lineage (CD4⁺ or CD8⁺) and subset (naïve or TEM) were pooled together. For all clones generated by the same VJ cassette pair diversity was computed by Shannon entropy (Fig. 4C and see methods). Across different VJ pairs, the Shannon entropy of naïve CD4⁺ and CD8⁺ T cell repertoires was close to the maximum possible diversity, $\log_2 N$, where N is the number of clonotypes generated by a particular VJ pair. By contrast, the VJ entropy of TEM samples was much lower than that of naïve T cells and was significantly reduced compared

to the maximum. Together, the diversity and VJ entropy of naïve and TEM cells in lymphoid sites indicates that naïve T cell clones exhibit considerably less clonal expansion compared to TEM clones, with naïve T cell clonal expansions observed more frequently in individuals > 40 years of age.

Lymphoid tissues share very few naïve clones regardless of donor age

A great advantage of our samples from organ donors is the ability to compare clonal distribution of naïve (or TEM) subsets between distinct sites, enabling an assessment of whether clonal expansions observed with naïve T cells resulted in sharing of specific clones between tissues sites. We assessed sequence overlap among the top 1000 clones for naïve and TEM cells from each of three tissues within individuals of different ages. (Actual numbers were slightly greater than 1000 to account for multiple clones observed with the same read count.) As previously reported (7) and consistent in additional donors examined of all ages (Fig. 5A and Fig. S7), there was appreciable overlap in TEM clones between sites with 20-30% of CD4 TEM clones and >40% of CD8 TEM clones found in more than one tissue site. By contrast, there were remarkably few naïve T cell clones found in more than one site, with the vast majority of naïve T cell clones unique to either spleen, ILN or LLN from the same donor (Fig. 5A). This minimal sharing of naïve T cell clones between tissue sites was observed in all donors examined independent of donor age (Fig. 5A and Fig. S7).

Given that naïve T cell clones were present in reduced overall frequencies compared to TEM cells, it was important to establish that the lack of overlap of naïve T cell clones between sites was not due to sampling or other quantitative differences in clone frequency. We therefore investigated how the fraction of clonal overlap between two tissues was related to the clonal read count or frequency for naïve and TEM cells for each donor (Fig. 5B). In the two representative donors, the overlap frequency for CD4⁺ and CD8⁺ TEM cells is low but measurable for clones with lower read counts, following a steep linear increase after a certain read count, with the most expanded clones having a high probability of being detected in both tissues (Fig. 5B, blue curve). By contrast, the clonal overlap versus read count curve for naïve T cells shows a greatly reduced association compared to TEM cells and differs as a function of age and for CD4⁺ versus CD8⁺ T cells (Fig. 5B,C). For naïve CD4⁺ T cells, clonal overlap is negligible for all read counts in the younger and older donor, showing a flat line (Fig. 5B), indicating minimal or negligible clonal overlap. For naïve CD8⁺ T cells, higher clone frequencies are associated with clonal overlap for the 21 year old donor, but not for the 51 year old donor (Fig. 5B). More strikingly, in the 51 year old donor, the expanded naïve T cell clones did not exhibit overlap between sites even when compared to a similar quantitative expansion of memory T cell clones (Fig. 5B, lower). When all data were compiled from 14 donors, minimal tissue sharing between expanded populations of naïve T cell clones is even more apparent, particularly for CD4⁺ T cells (Fig. 5C). Together, these quantitative analyses reveals an unexpected compartmentalization of expanded populations of naïve CD4⁺ and CD8⁺ T cells in lymphoid sites, suggesting *in situ* expansion during their maintenance *in vivo*.

We also sequenced naïve and TEM cells from replicate samples to calculate the detection power at a given frequency (See methods and Table S6). Clonal overlap was calculated for

each set of replicates and for corresponding non-replicate tissue samples from the same donors. For every read count, the ratio between the fraction of clones shared among replicates was compared to the fraction shared between different tissues to obtain an average overlap rate. This analysis yielded rates between 0.1-0.3 for naïve CD4⁺ T cells, 0.3-0.5 for naïve CD8⁺ T cells, 0.6-0.75 for TEM CD4⁺ T cells and 0.6-0.9 for TEM CD8⁺ T cells (Table S6). Compared to baseline overlap frequencies from the replicate analysis, the negligible frequency of inter-tissue overlap of naïve T cell clones is still consistent with *in situ* maintenance.

Distribution of VJ cassette use among naïve T cells becomes more dissimilar with age

We hypothesized that the distribution of VJ combinations would be similar between T cells in different sites when seeded by the thymus, but may diverge when maintained independent of thymic output. We tested this using the Jensen Shannon Distance (JSD, see methods) (29). The JSD is a measure of the distance between two probability distributions, with two identical distributions having 0 distance, and two maximally different distributions having distance 1. The VJ distribution for the 21-year old donor is similar for both SP and LLN, and hence the value of JSD is low (Fig. 6A, top). However, VJ usage for these tissues in a 51 year old exhibits many differences, and consequently the inter-tissue distance is significantly larger (Fig. 6A, lower). Computing JSD for naïve T cells in tissue pairs for each donor reveals a clear increase in VJ distance among older donors for CD4⁺ T cells, with a similar trend for CD8⁺ T cells, consistent with biased expansion and maintenance of naïve T cells in specific sites (Fig. 6B).

Discussion

The maintenance of naïve T cells over a lifetime ensures that the immune system can respond to new antigens not previously encountered, reducing the likelihood of succumbing to infectious pathogens at different life stages. The extent to which naïve T cells are maintained in humans and the mechanisms for their longterm persistence have proved difficult to address based on the limited sampling of peripheral blood. Here, we took a new approach to analyze naïve T cell development, maintenance and repertoire in primary and secondary lymphoid sites from 128 organ donors spanning over 7 decades of life. Our findings identify lymph nodes as major reservoirs for maintenance of naïve T cells and a diverse TCR repertoire in the presence of waning thymic output which is markedly diminished after age 40. We further reveal tissue compartmentalization and *in situ* homeostasis as new mechanisms for preserving naiveté in the human T cell compartment.

Age-associated changes in the thymus are well documented and include reduction of thymic volume, loss of thymic epithelial cells, increase in the perivascular space and predominance of adipose tissue (15, 30, 31). From our analysis of thymic tissue of different ages, double positive CD4⁺CD8⁺ thymocytes were 60-80% of total thymocytes up until the fifth decade of life, after which there is a significant reduction to 5-15% DP cells. While this overall conclusion that precipitous changes in thymic output are occurring in middle age has not previously been emphasized, low and variable DP frequencies and reductions in thymocyte number were previously reported in adult compared to pediatric thymii (32, 33). The precise

mechanisms for this decline in thymopoiesis is unclear, but could be due to alterations in thymic epithelial cells, as expression of the FoxN1 transcription factor required for thymic function in mice (34, 35) was recently found reduced in adult thymii (36).

This steep reduction in thymic output after 40 years of age also paralleled the reduction in TREC levels (as indicators of RTE) in naïve T cells from spleen and two lymph node sites. Previous studies showed reduced TREC levels with age in peripheral blood naïve T cells, with 10-fold reductions in TREC levels between the third and fifth decade of life (8, 37). Here, we reveal differential TREC content in lymphoid tissue naïve T cells in younger individuals (<40 years of age), but equivalent TREC levels between tissues in older individuals (>40 years of age). In mouse models, RTE become mature naïve T cells upon entry into LN (38), suggesting that unequal TREC content in tissues may indicate active thymic output to certain sites. Together, our results suggest an age-related program involved in cessation of thymic output during the middle years of life.

Despite these reductions in thymic export, 20-40% of T cells in lymph nodes (and lower percentages in spleen) are maintained as phenotypically and functionally naïve. To dissect mechanisms for this long-term persistence of naïve T cells, we used deep sequencing of TCR β chains in naïve T cells in spleen, ILN and LLN of donors from 1-60 years. Integrating naïve TCR results from all donors and all sites reveals that TCR diversity of naïve T cells is largely maintained in tissues, with clonal expansions of specific naïve T cell clones observed mostly in donors >40 years of age, but still much reduced compared to that observed with memory T cells. This modest loss of diversity of naïve T cells with age has also been reported by deep sequencing of TCR of naïve T cells in blood (4). Thus, human naïve T cells in tissues preserve both their diversity and functionality independent of thymic output.

More strikingly, and unexpectedly, we found minimal overlap of expanded naïve T cell clones between individual lymphoid sites in donors of all ages, especially for CD4⁺ cells. Overlap of highly expanded clones of naïve-phenotype CD8⁺ T cells were detected; however, subsets of human primed CD8⁺ T cells can exhibit naïve phenotypes (39, 40). By comparing clonal overlap as a function of clone frequency, we demonstrate that for a given clone frequency, the majority of naïve T cell clones are detected in a single site while memory T cell clones are detected in multiple sites. These results suggest two possible mechanisms for naïve T cell maintenance: First, that naïve T cells remain resident in specific sites and do not readily migrate between tissues, and second, that clonal expansions are occurring within specific sites through *in situ* signals, particularly for older donors with negligible thymic output.

In contrast to our finding of specific clones of naïve T cells being specific to a tissue site, it is generally understood from mouse studies that naïve T cells continuously recirculate between lymphoid sites, lymph and blood (41). In mice, adoptive transfer of naïve T cells results in dissemination to multiple secondary lymphoid organs (42) via CCR7 expression (43). However, mouse naïve T cells require cognate interactions of the TCR with MHC molecules for survival and/or functional maintenance (44) and homeostatic cytokines such as IL-7 (45, 46)—interactions which are more likely to occur in LN and not during transit through circulation.

It is now recognized that a substantial proportion of mouse (and human) memory T cells can be retained in tissues as tissue-resident memory T cells (TRM) distinguished by CD69 expression which is also a marker of early T cell activation (7, 12, 13, 47). However, tissue residence has not been previously associated with naïve T cells. We found that in human tissues, ~20% of naïve T cells upregulate CD69 indicating signaling or potentially transient retention in lymph node tissue sites. As mouse naïve T cells do not exhibit CD69 upregulation and are largely maintained by thymic output throughout life (8), it is possible that their migration behaviors may be distinct from those of long-lived human naïve T cells exhibiting peripheral homeostatic expansion. We propose that in humans, retention of naïve T cells in lymph nodes may be required for their long-term preservation.

The site-specific clonal expansion of naïve T cells identified here suggests a non-random nature to naïve T cell persistence in a particular site. Elegant studies in mice showed that naïve CD4⁺ T cell survival was linked to their clonal abundance and specificity (48, 49). As human naïve T cells need to persist for decades, we propose that cognate interactions may determine which naïve T cell clones get retained or survive in a particular site. This bias in naïve T cell specificity in different sites is supported by our VJ usage difference results revealing a distinct repertoire skewing in one lymphoid site compared to another that was most striking after cessation of thymic output. These results indicate a biased expansion of specific clones in tissue sites.

While our study provides a “snap shot” of human T cell subset composition within lymphoid and mucosal sites in individuals over a broad age range that spans 7 decades of life, there are caveats of our study to be considered. We did not specifically examine antigen- or pathogen-specific responses. The maintenance of antigen-specific naïve T cells may follow different kinetics or dynamics from the phenotypic naïve subsets examined here. There are caveats to the TCR clonal analysis, which are based on sequences obtained from a fraction of the total naïve T (or TEM) cells in a particular tissue—it was not possible to sequence every single T cell in human spleen, for example, due to the impracticality of this endeavor. We used bioinformatics analysis and calculations of frequency to determine clonal overlap and cannot rule out that there are clones that were not captured by our analysis that may exhibit different behavior with regard to tissue overlap.

In conclusion, our investigations of T cells in human primary and secondary lymphoid organs has revealed new insights into naïve T cell maintenance that cannot be extrapolated from sampling of peripheral blood. Our findings provide alternative mechanisms for the conservation of the naïve immune response by *in situ* homeostasis and maintenance in lymphoid tissues that may be specific to humans. These results showing site-specific repertoires for naïve T cells have implications for the design of vaccines and immunotherapies for promoting and regulating immune response, particularly in the middle years and beyond

Materials and Methods

Study Design

Research Objectives—To examine naïve T cell longevity and mechanisms for maintenance and correlate to thymic output in human lymphoid organs.

Research subjects—Tissue samples from organ donors aged 2 months-73 years.

Experimental design—Lymphocytes were isolated from tissues, and CD4⁺ and CD8⁺ T cell subsets were analyzed by flow cytometry for phenotypic parameters, by histology, cytokine production, PCR for detection of T cell receptor excisions circles (TREC), and DNA sequencing of TCR genes.

Randomization—Tissues were obtained from brain-dead organ donors in the NY metropolitan area and were obtained as available. All donor data obtained were included in this study. Results were not blinded.

Sample Size—Data are compiled from tissues from 128 organ donors aged 2 months-73 years. The number of donors analyzed for each different experimental approach is indicated the figure legends and text.

Acquisition of Human Tissues

Human tissues were obtained from deceased (brain dead) organ donors at the time of organ acquisition for clinical transplantation through an approved research protocol and MTA with LiveOnNY. All donors were free of chronic disease and cancer, were Hepatitis B, C, and HIV-negative (Table S1). Tissues were collected after the donor organs were flushed with cold preservation solution and clinical procurement process was completed. Acquisition of these samples does not qualify as “human subjects” research, as confirmed by the Columbia University IRB, as tissues were obtained from deceased individuals. In some cases, thymus tissue was also obtained as discarded tissue from patients undergoing pediatric cardiac surgery through the Human Studies Core of the Columbia Center for Translational Immunology (CCTI), with IRB approvals maintained by this core. Thymus tissue was removed by trained cardiothoracic surgeons during organ donor acquisition and thymus tissue was further confirmed by H&E staining.

Lymphocyte isolation from tissue sites

Tissue samples were maintained in cold saline and brought to the laboratory within 2-4 hours of organ procurement. Samples were rapidly processed using enzymatic and mechanical digestion as previously described (11, 12, 50), resulting in high yields of viable lymphocytes.

Thymus histology

Thymus tissue samples obtained from donors as above were cryopreserved and fixed in optimum cutting temperature (OCT) matrix (Tissue-Tek) and maintained at -80°C before sectioning. Sections were stained with hematoxylin and eosin (H&E) by the Histology Core

service within the Department of Pathology, Columbia University Medical Center. The presence and number of Hassall Corpuscles were counted based on structures per viewing frame at 10× magnification in three separate tissue sections.

Flow Cytometry Analysis and sorting

For analysis of cell-surface markers via flow cytometry, single-cell suspensions were stained with fluorochrome-conjugated antibodies in flow cytometry staining buffer (1% FBS/0.1% sodium azide in PBS) presented in Table S5. Control samples included unstained and single fluorochrome-stained compensation beads (OneComp ebeads, eBioscience). Stained cells were analyzed using a LSRII or FACSCanto flow cytometer (BD Biosciences) with FACSDiva (BD Biosciences) and were analyzed using FlowJo software (Tree Star). For isolation of subsets, T cells stained as above were sorted using a BD-influx (BD Biosciences), with single cell compensation controls acquired as above. Representative gating strategies used for the thymocytes and T cell subsets is in Fig. S1.

T cell stimulation and cytokine analysis

T cells were cultured in 96 well plates (100,000 cells/well) +/- anti-CD2/CD3/CD28 coated beads (1 bead:1 cell) for two days in Clicks media at 37°C. Supernatants from T cell cultures were analyzed for cytokine content by cytometric bead array (CBA) using the BD Biosciences Human Th1/Th2 cytokine kit II. Standard analytes were acquired on the BD LSR-II flow cytometer using provided templates and a standard curve was generated to calculate sample concentration.

Quantification of human T cell receptor excision circles (TREC)

Naïve (CCR7⁺/CD45RA⁺) CD4⁺ and CD8⁺ T cells were sorted from spleen, ILN, LLN, and MLN of individual donors, single positive CD4⁺ and CD8⁺ thymocytes were sorted from thymus tissues, and cell pellets were stored at -80°C prior to analysis. TREC content was quantified using an established real-time PCR approach with a standard curve of known molecules of human TREC (51). The following primer and probe sequences were used:

*5' primer= 5'-CACATCCCTTTCAACCATGCT-3'

3' primer= 3'-GCCAGCTGCAGGGTTTAGG-3'

probe= 5'-6-FAM-CAGGGCAGGTTTTGTAAAGGTGCTCACTT-3' BHQ1 (BHQ=Black Hole Quencher)

Statistical analysis and data visualization for cellular data

Descriptive statistics (means, standard deviations, counts) were calculated for each T cell subset and tissue in Microsoft Excel. Frequency variance was determined for each subset and tissue by Holm-Sidak post-hoc multiple comparison following two-way ANOVA to exclude subset-dependent effects in GraphPad PRISM (Graphpad software, Inc.). Resulting two-tailed p-values and r-values were graphed in Microsoft Excel and using GraphPad Prism.

T cell receptor sequencing

Naïve (CD45RA⁺CCR7⁺) and TEM (CD45RA⁺CCR7⁻) -phenotype CD4⁺ and CD8⁺ T cells were sorted from 2-3 whole lymph nodes for ILN and LLN and human spleen (7cm² pieces) from individual donors (Table S1). In some cases, DNA was isolated from cell pellets using the AllPrep DNA/RNA mini kit (Qiagen) in conjunction with QIAshredder columns (Qiagen), and DNA concentration was assessed using the NanoDrop (Thermo Scientific). Either cell pellets or DNA was sent to Adaptive Biotechnologies for TCRβ deep sequencing using the ImmunoSEQ™ platform (24). Cell number and productive reads for each population from each donor are presented in Table S5.

TCR data acquisition and quality control

The ImmunoSEQ™ platform used for TCR sequencing has a standardized protocol that uniquely identifies and amplifies the CDR3, using spiked in synthetic templates and clustering techniques to correct for PCR and sequencing errors (24, 27). The resultant sequencing data were downloaded from Adaptive servers (nucleotide, amino acid, V and J genes, and read counts). Datasets were filtered to select for productive sequences (in-frame, absent any premature stop codons) using the “frametype” designation (Adaptive). We verified data quality by subsampling our data and identifying plateaus in the number of unique clones observed, indicating saturation of sequenced chromosome fragments.

To identify contaminating clones within a donor due to sorting (which is 99% accurate), we applied a filter to remove low-level contamination between CD4⁺ and CD8⁺ samples. For each clone observed in any of the samples in a donor, we assigned CD4⁺ and CD8⁺ identity based on maximum frequency (p) among all samples from the same donor. For a clone assigned as CD4⁺, if it was present in any of the CD8⁺ samples from the same donor with frequency $< 0.5p$, then this clone was removed from the CD8⁺ samples as a contaminant from CD4⁺ (and vice-versa); otherwise, this clone was determined to be ambiguous and was removed from all samples. On average, the fraction of reads which were filtered from naïve samples was $< 0.4\%$ and $< 1.5\%$ from TEM samples (Fig. S8),

Statistical methods for analyzing TCR sequencing data

Clonal diversity was measured using Simpson's index and entropy. Simpson's index was used to quantitate diversity for naïve T cells which exhibited low clonal expansion, and is defined as the sum of the squared clonal frequencies:

$$SI = \sum_{i=1}^N p_i^2$$

Shannon entropy was used to compare diversity of clones from each type of VJ gene combination. Entropy is defined as the negative expected value of the log of observed clonal frequencies,

$$H = - \sum_{i=1}^N p_i \log_2 p_i$$

and provides a balance between species richness (number of unique sequences) and evenness (their frequency in the population); it is closely related to the Jensen Shannon Distance used in divergence calculations (52) which is given by the following equation:

$$JSD(P, Q) = \sqrt{H\left(\frac{1}{2}[P+Q]\right) - \frac{1}{2}[H(P)+H(Q)]}$$

Jensen Shannon distance was computed for each donor between every pair of tissues P and Q, as previously described (52). Distance was computed for VJ gene pairs. Calculations were performed in R.

Measuring clonal overlap between tissue sites

The top 1000 clones were selected by nucleotide sequence, with additional clones included if they were present at the same read count as the thousandth clone in the sample after sorting by read count. Clonal overlap was measured as the number of nucleotide sequences found in all samples being compared. Overlap between pairs of tissues across frequency was computed in R, with read count of clones in tissue 1 present on the x-axis and frequency of overlap with all clones of tissue 2 on the y-axis. In order to resolve cases with only a few clones observed at larger counts resulting in overlap frequencies of 0 or 1, clones present at read counts greater than 20 in tissue 1 were grouped into 25 bins of equal size on the log10 scale. Identical sequences from different donors were kept separate to avoid changing read counts corresponding to a sample. The same approach was then applied to the aggregated data across the two tissues.

To correct for variability in detection power of shared clones due to differences in the average clonal frequency, we used replicate sharing as a baseline for inter-tissue sharing (See Table S6). For every read count, we compared the fraction (f1) of overlapping clones observed between two sites with the fraction (f0) of overlapping clones from replicates, defining overlap rate as $r = f1/f0$ for naïve and TEM samples. An average ratio over all read counts was then computed, weighted by the number of clones present in each bin to adjust for detection power.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Aw D, Palmer DB. The origin and implication of thymic involution. *Aging Dis.* 2011; 2:437–443. [PubMed: 22396892]
2. Evans CJ, Ho Y, Daveson BA, Hall S, Higginson IJ, Gao W. G. U. C. project. Place and cause of death in centenarians: a population-based observational study in England, 2001 to 2010. *PLoS Med.* 2014; 11:e1001653. [PubMed: 24892645]
3. Chinn IK, Blackburn CC, Manley NR, Sempowski GD. Changes in primary lymphoid organs with aging. *Semin Immunol.* 2012; 24:309–320. [PubMed: 22559987]
4. Qi Q, Liu Y, Cheng Y, Glanville J, Zhang D, Lee JY, Olshen RA, Weyand CM, Boyd SD, Goronzy JJ. Diversity and clonal selection in the human T-cell repertoire. *Proc Natl Acad Sci U S A.* 2014; 111:13139–13144. [PubMed: 25157137]
5. Cossarizza A, Ortolani C, Paganelli R, Barbieri D, Monti D, Sansoni P, Fagiolo U, Castellani G, Bersani F, Londei M, Franceschi C. CD45 isoforms expression on CD4+ and CD8+ T cells throughout life, from newborns to centenarians: implications for T cell memory. *Mech Ageing Dev.* 1996; 86:173–195. [PubMed: 8733112]
6. Farber DL, Yudanin NA, Restifo NP. Human memory T cells: generation, compartmentalization and homeostasis. *Nat Rev Immunol.* 2014; 14:24–35. [PubMed: 24336101]
7. Thome JJC, Yudanin NA, Ohmura Y, Kubota M, Grinshpun B, Sathaliyawala T, Kato T, Lerner H, Shen Y, Farber DL. Spatial Map of Human T Cell Compartmentalization and Maintenance over Decades of Life. *Cell.* 2014; 159:814–828. [PubMed: 25417158]
8. den Braber I, Mugwagwa T, Vrisekoop N, Westera L, Mogling R, de Boer AB, Willems N, Schrijver EH, Spierenburg G, Gaiser K, Mul E, Otto SA, Ruiter AF, Ackermans MT, Miedema F, Borghans JA, de Boer RJ, Tesselaar K. Maintenance of peripheral naive T cells is sustained by thymus output in mice but not humans. *Immunity.* 2012; 36:288–297. [PubMed: 22365666]
9. Goronzy JJ, Fang F, Cavanagh MM, Qi Q, Weyand CM. Naive T Cell Maintenance and Function in Human Aging. *J Immunol.* 2015; 194:4073–4080. [PubMed: 25888703]
10. Ganusov VV, De Boer RJ. Do most lymphocytes in humans really reside in the gut? *Trends Immunol.* 2007; 28:514–518. [PubMed: 17964854]
11. Thome JJ, Bickham KL, Ohmura Y, Kubota M, Matsuoka N, Gordon C, Granot T, Griesemer A, Lerner H, Kato T, Farber DL. Early-life compartmentalization of human T cell differentiation and regulatory function in mucosal and lymphoid tissues. *Nat Med.* 2016; 22:72–77. [PubMed: 26657141]
12. Thome JJ, Farber DL. Emerging concepts in tissue-resident T cells: lessons from humans. *Trends Immunol.* 2015; 36:428–435. [PubMed: 26072286]
13. Schenkel JM, Masopust D. Tissue-Resident Memory T Cells. *Immunity.* 2014; 41:886–897. [PubMed: 25526304]
14. Lynch HE, Goldberg GL, Chidgey A, Van den Brink MR, Boyd R, Sempowski GD. Thymic involution and immune reconstitution. *Trends Immunol.* 2009; 30:366–373. [PubMed: 19540807]
15. Haynes BF, Markert ML, Sempowski GD, Patel DD, Hale LP. The role of the thymus in immune reconstitution in aging, bone marrow transplantation, and HIV-1 infection. *Annu Rev Immunol.* 2000; 18:529–560. [PubMed: 10837068]
16. Raica M, Encica S, Motoc A, Cimpean AM, Scridon T, Barsan M. Structural heterogeneity and immunohistochemical profile of Hassall corpuscles in normal human thymus. *Ann Anat.* 2006; 188:345–352. [PubMed: 16856599]

17. Spits H. Development of alphabeta T cells in the human thymus. *Nat Rev Immunol.* 2002; 2:760–772. [PubMed: 12360214]
18. Vanhecke D, Leclercq G, Plum J, Vandekerckhove B. Characterization of distinct stages during the differentiation of human CD69+CD3+ thymocytes and identification of thymic emigrants. *J Immunol.* 1995; 155:1862–1872. [PubMed: 7543535]
19. Hazenberg MD, Borghans JA, de Boer RJ, Miedema F. Thymic output: a bad TREC record. *Nat Immunol.* 2003; 4:97–99. [PubMed: 12555089]
20. Hazenberg MD, Otto SA, de Pauw ES, Roelofs H, Fibbe WE, Hamann D, Miedema F. T-cell receptor excision circle and T-cell dynamics after allogeneic stem cell transplantation are related to clinical events. *Blood.* 2002; 99:3449–3453. [PubMed: 11964316]
21. Hazenberg MD, Verschuren MC, Hamann D, Miedema F, van Dongen JJ. T cell receptor excision circles as markers for recent thymic emigrants: basic aspects, technical approach, and guidelines for interpretation. *J Mol Med.* 2001; 79:631–640. [PubMed: 11715066]
22. Sempowski G, Thomasch J, Gooding M, Hale L, Edwards L, Ciafaloni E, Sanders D, Massey J, Douek D, Koup R, Haynes B. Effect of thymectomy on human peripheral blood T cell pools in myasthenia gravis. *J Immunol.* 2001; 166:2808–2817. [PubMed: 11160348]
23. Kimmig S, Przybylski GK, Schmidt CA, Laurisch K, Mowes B, Radbruch A, Thiel A. Two subsets of naive T helper cells with distinct T cell receptor excision circle content in human adult peripheral blood. *J Exp Med.* 2002; 195:789–794. [PubMed: 11901204]
24. Robins H. Immunosequencing: applications of immune repertoire deep sequencing. *Curr Opin Immunol.* 2013; 25:646–652. [PubMed: 24140071]
25. Robins HS, Campregher PV, Srivastava SK, Wachter A, Turtle CJ, Kahsai O, Riddell SR, Warren EH, Carlson CS. Comprehensive assessment of T-cell receptor beta-chain diversity in alphabeta T cells. *Blood.* 2009; 114:4099–4107. [PubMed: 19706884]
26. Gaide O, Emerson RO, Jiang X, Gulati N, Nizza S, Desmarais C, Robins H, Krueger JG, Clark RA, Kupper TS. Common clonal origin of central and resident memory T cells following skin immunization. *Nat Med.* 2015; 21:647–653. [PubMed: 25962122]
27. Hsu MS, Sedighim S, Wang T, Antonios JP, Everson RG, Tucker AM, Du L, Emerson R, Yusko E, Sanders C, Robins HS, Yong WH, Davidson TB, Li G, Liao LM, Prins RM. TCR Sequencing Can Identify and Track Glioma-Infiltrating T Cells after DC Vaccination. *Cancer Immunol Res.* 2016; 4:412–418. [PubMed: 26968205]
28. Morris H, De Wolf S, Robins H, Sprangers B, Lo Cascio SA, Shonts BA, Kawai T, Wong W, Yang S, Zuber J, Shen Y, Sykes M. Tracking donor-reactive T cells: Evidence for clonal deletion in tolerant kidney transplant patients. *Sci Transl Med.* 2015; 7:272ra210.
29. Sims JS, Grinshpun B, Feng Y, Ung TH, Neira JA, Samanamud JL, Canoll P, Shen Y, Sims PA, Bruce JN. Diversity and divergence of the glioma-infiltrating T-cell receptor repertoire. *Proceedings of the National Academy of Sciences of the United States of America.* 2016; 113:E3529–3537. [PubMed: 27261081]
30. Flores KG, Li J, Sempowski GD, Haynes BF, Hale LP. Analysis of the human thymic perivascular space during aging. *J Clin Invest.* 1999; 104:1031–1039. [PubMed: 10525041]
31. Taub DD, Longo DL. Insights into thymic aging and regeneration. *Immunol Rev.* 2005; 205:72–93. [PubMed: 15882346]
32. Jamieson BD, Douek DC, Killian S, Hultin LE, Scripture-Adams DD, Giorgi JV, Marelli D, Koup RA, Zack JA. Generation of functional thymocytes in the human adult. *Immunity.* 1999; 10:569–575. [PubMed: 10367902]
33. Shiraishi J, Utsuyama M, Seki S, Akamatsu H, Sunamori M, Kasai M, Hirokawa K. Essential microenvironment for thymopoiesis is preserved in human adult and aged thymus. *Clin Dev Immunol.* 2003; 10:53–59. [PubMed: 14575158]
34. Bredenkamp N, Nowell CS, Blackburn CC. Regeneration of the aged thymus by a single transcription factor. *Development.* 2014; 141:1627–1637. [PubMed: 24715454]
35. Bredenkamp N, Ulyanchenko S, O'Neill KE, Manley NR, Vaidya HJ, Blackburn CC. An organized and functional thymus generated from FOXN1-reprogrammed fibroblasts. *Nat Cell Biol.* 2014; 16:902–908. [PubMed: 25150981]

36. Reis MD, Csomos K, Dias LP, Prodan Z, Szerafin T, Savino W, Takacs L. Decline of FOXP1 gene expression in human thymus correlates with age: possible epigenetic regulation. *Immun Ageing*. 2015; 12:18. [PubMed: 26516334]
37. Douek DC, McFarland RD, Keiser PH, Gage EA, Massey JM, Haynes BF, Polis MA, Haase AT, Feinberg MB, Sullivan JL, Jamieson BD, Zack JA, Picker LJ, Koup RA. Changes in thymic function with age and during the treatment of HIV infection. *Nature*. 1998; 396:690–695. [PubMed: 9872319]
38. Fink PJ, Hendricks DW. Post-thymic maturation: young T cells assert their individuality. *Nat Rev Immunol*. 2011; 11:544–549. [PubMed: 21779032]
39. Pulko V, Davies JS, Martinez C, Lanteri MC, Busch MP, Diamond MS, Knox K, Bush EC, Sims PA, Sinari S, Billheimer D, Haddad EK, Murray KO, Wertheimer AM, Nikolich-Zugich J. Human memory T cells with a naive phenotype accumulate with aging and respond to persistent viruses. *Nat Immunol*. 2016
40. Fuertes Marraco SA, Soneson C, Cagnon L, Gannon PO, Allard M, Abed Maillard S, Montandon N, Rufer N, Waldvogel S, Delorenzi M, Speiser DE. Long-lasting stem cell-like memory CD8+ T cells with a naive-like profile upon yellow fever vaccination. *Sci Transl Med*. 2015; 7:282ra248.
41. Takada K, Jameson SC. Naive T cell homeostasis: from awareness of space to a sense of place. *Nat Rev Immunol*. 2009; 9:823–832. [PubMed: 19935802]
42. Reinhardt RL, Khoruts A, Merica R, Zell T, Jenkins MK. Visualizing the generation of memory CD4 T cells in the whole body. *Nature*. 2001; 410:101–105. [PubMed: 11242050]
43. Bromley SK, Thomas SY, Luster AD. Chemokine receptor CCR7 guides T cell exit from peripheral tissues and entry into afferent lymphatics. *Nat Immunol*. 2005; 6:895–901. [PubMed: 16116469]
44. Surh CD, Sprent J. Homeostasis of naive and memory T cells. *Immunity*. 2008; 29:848–862. [PubMed: 19100699]
45. Tan JT, Dudl E, LeRoy E, Murray R, Sprent J, Weinberg KI, Surh CD. IL-7 is critical for homeostatic proliferation and survival of naive T cells. *Proc Natl Acad Sci U S A*. 2001; 98:8732–8737. [PubMed: 11447288]
46. Schluns KS, Kieper WC, Jameson SC, Lefrancois L. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nature Immunology*. 2000; 1:426–432. [PubMed: 11062503]
47. Teijaro JR, Turner D, Pham Q, Wherry EJ, Lefrancois L, Farber DL. Cutting edge: tissue-retentive lung memory CD4 T cells mediate optimal protection to respiratory virus infection. *J Immunol*. 2011; 187:5510–5514. [PubMed: 22058417]
48. Jenkins MK, Chu HH, McLachlan JB, Moon JJ. On the composition of the preimmune repertoire of T cells specific for Peptide-major histocompatibility complex ligands. *Annu Rev Immunol*. 2010; 28:275–294. [PubMed: 20307209]
49. Hataye J, Moon JJ, Khoruts A, Reilly C, Jenkins MK. Naive and memory CD4+ T cell survival controlled by clonal abundance. *Science*. 2006; 312:114–116. [PubMed: 16513943]
50. Sathaliyawala T, Kubota M, Yudanin N, Turner D, Camp P, Thome JJ, Bickham KL, Lerner H, Goldstein M, Sykes M, Kato T, Farber DL. Distribution and compartmentalization of human circulating and tissue-resident memory T cell subsets. *Immunity*. 2013; 38:187–197. [PubMed: 23260195]
51. Sempowski GD, Gooding ME, Liao HX, Le PT, Haynes BF. T cell receptor excision circle assessment of thymopoiesis in aging mice. *Mol Immunol*. 2002; 38:841–848. [PubMed: 11922942]
52. Sims JS, Grinshpun B, Feng Y, Ung TH, Neira JA, Samanamud JL, Canoll P, Shen Y, Sims PA, Bruce JN. Diversity and divergence of the glioma-infiltrating T-cell receptor repertoire. *Proc Natl Acad Sci U S A*. 2016

Fig. 1. Alterations in Thymus structure and diminished thymopoiesis with age

(A) Representative histology of thymus sections from individuals of indicated ages visualized by H&E staining shown 10× magnification (top, scale bars of 200μm indicated at lower left), with an enlarged section shown below at 40× magnification (scale bars of 50μm at lower left). Hassall Corpuscle structures are denoted by red arrows in each field. (B) Average Number of Hassall Corpuscle structures per viewing pane in pediatric (0-2 years, n=8, white), and adult (n=6, aged 23,25,30,49,50,57 years, black) donors. P-value denoted by **=0.0002. (C) *Left*: Representative flow cytometry plots indicating the coordinate expression of CD4 and CD8 on T cells in thymus tissue from donors of indicated ages, delineating double positive (DP) and CD4 and CD8 single positive (SP) subsets with percent of each indicated in quadrants. *Right*: CD69 expression by DP and SP subsets from thymus tissue obtained from donors of indicated ages. (D) Compiled percentage of DP thymocytes in thymus tissue of donors of indicated ages and gender (blue, male; red, female) from 27 donors (See Table S1).

Fig. 2. Naïve CD4⁺ and CD8⁺T cells are differentially maintained in lymphoid sites
 (A) Mean frequencies $\pm$ SEM of naïve (CCR7⁺CD45RA⁺) CD4⁺ (left) and CD8⁺ (right) T cells in indicated tissues from donors stratified into 3 age groups: pediatric (0-2 years, red, n=18), younger adults (15-40 years, black dashed, n=27), and older adults (41+ years, black, n=24) donors. See individual means, SEM and p values in Table S2. (B) Naïve CD4⁺ (top) or CD8⁺ (bottom) within four indicated lymphoid tissue sites as a function of age with each dot representing an individual donor compiled from 69 donors (see Table S1). Average CD8:CD4 ratios SP, 31:35, p=0.16; ILN, 57:42, p<0.001; LLN, 49:38, p<0.001; MLN, 48:41, p=0.01) (C) Naïve T cell frequency in multiple tissue of individual donors (total=40) where all four tissue sites were obtained, shown as a heat map of highest and lowest percent naïve T cells ranked by age in donors

Fig. 3. Naïve T cells retain functionality independent on the extent of recent thymic emigrants (RTE)

(A) RTE content among naïve T cells was measured by quantitative of T cell receptor excision circles (TREC) using a PCR-based approach (See online methods). Graphs show TREC content per 100,000 sorted CD4⁺ (top) and CD8⁺ (bottom) naïve T cells from indicated lymphoid sites (spleen, circle; ILN, upside down triangle; LLN, square; MLN, triangle), SP cells from each thymus (indicated by “X”) and total T cells from tissues obtained from the 17yr old and 73yr old donors. Each color represents an individual donor (individual values in Table S3). (B) Naïve and TEM CD4⁺ and CD8⁺ T cells were sorted from spleen, ILN and MLN, stimulated with anti CD3/CD28/CD2 beads, and the cytokine content in supernatants was assessed using the BD Cytokine Bead Array kit (see methods). Shown are IL-2 and IFN- γ production (pg/ml, mean $\pm$ SEM) by naïve CD4 and CD8 T cells isolated from tissues of donors under age 35yrs of age (white bars, 2-4 donors) and over 50yrs of age (black bars, 2-4 donors except for spleen CD4 T cells which is from 1 donor). (C) IL-2 and IFN- γ production by naïve and memory (TEM) CD4 and CD8 T cells from the LLN of individual donors <35 years (4 donors: 29, 25, 26, and 34 years) and >50 years (4 donors: 54, 56, 52, and 59 years) of age. Cytokine levels are normalized by donor and indicated by Z score ((cytokine level-mean cytokine level)/standard deviation). Values for each cytokine measured are shown in Table S4.

Fig. 4. TCR Repertoire diversity of naïve T cells decreases with age and is distinct from TEM subsets

Naïve (CD45RA⁺CCR7⁺) CD4⁺ and CD8⁺ T cells were sorted from spleen, inguinal and mesenteric lymph nodes (ILN, MLN) and CDR3 β sequences were amplified and sequenced (See methods). (A) Repertoire diversity within spleen (red), ILN (blue) and LLN (green) tissues is quantified in bulk by Simpson's index (see methods) and plotted against donor age, as shown for CD4⁺ (left) and CD8⁺ (right) lineages compiled from 11 and 13 donors, respectively (see Table S3), with each dot representing a single tissue from an individual donor. (B) Maximum clone frequency is higher for memory compared to naïve T cells. The frequency of the largest sequenced clone in the sample is plotted for CD4⁺ and CD8⁺ T cells for both Naïve and TEM subsets. Significant p-values based on students' t-test are indicated between CD4⁺ and CD8⁺ TEM cells, and between Naïve and TEM populations within the CD4⁺ or CD8⁺ lineage. (C) Clonal diversity of every observed VJ combination as computed by Shannon entropy for naïve and TEM subsets for CD4⁺ (top) and CD8⁺ (bottom) lineages. Naïve populations are depicted by triangles, TEM populations by circles. Clones were pooled from every donor and separated by their VJ cassette. The curve log₂N depicts the maximum possible diversity for a fixed number of clones.

Fig. 5. Naïve repertoire exhibits minimal sharing between tissues

The nucleotide sequence of each clone in multiple tissues from individual donors was analyzed for clonal overlap. (A) Venn diagrams displaying nucleotide sequence overlap of the top 1000 clones naïve and TEM CD4⁺ (top) and CD8⁺ (bottom) clones between three tissues sites (Spleen, red; ILN, blue; LLN, green) of a 21year old (left, Donor 125) and a 51year old (right, Donor 201) donor. Actual numbers are slightly greater than 1000 to account for additional clones present in identical read numbers to the one thousandth clone. (B) Overlap of naïve (gold) or TEM (Blue) clones between tissue pairs as a function of clone frequency (quantified as read count) for the representative donors shown in (A). For clones with a given read count in the first tissue, the fraction of overlap with all clones in the second tissue is plotted. Counts greater than 20 are logarithmically binned into 25 bins. (C) Overlap versus clone frequency plot calculated as in (b) where for every tissue clones have been pooled from multiple donors analyzed. The number of donors is indicated in each individual graph.

Fig. 6. VJ usage between tissues shows divergence of the naïve T cell repertoire among older donors

(A) The top 50 VJ frequencies for CD4⁺ T cells from Spleen and LLN are plotted side by side for two representative donors, age 21 and age 51. Corresponding VJ distance for the sample is given in the top right corner. (B) The VJ-distance for 8 donors (7 CD4⁺, 7 CD8⁺). Distance is computed for every pair of tissues from the same donors and plotted against donor age. Distance between spleen and lung lymph node (white), inguinal lymph node and spleen (gray), lung lymph node and inguinal lymph node (black).