The maintenance of the naive T cell pool during homeostasis is differently regulated in humans and wildling mice

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Abstract

Understanding how naive T cells are maintained during healthy aging is important to unravel the effects of lymphopenic conditions on naive T cell dynamics. The human naive T cell compartment is thought to be mainly maintained through peripheral division, while naive T cells in specific pathogen-free (SPF) mice are mostly produced by the thymus. Previously, stem cell-like memory T cells (T_{SCM} cells) were included in the human naive T cell compartment. Because T_{SCM} cells, which increase in proportion throughout life, have a low T cell receptor excision circle (TREC) content compared to truly naive T cells (T_{TN} cells), the relative contribution of the thymus to the production of human naive T cells could have been underestimated. Here we confirm, through the measurement of TRECs, that the human T_{TN} cell compartment is almost exclusively maintained by peripheral division. Furthermore, we show that the thymus contributes more to the production of $CD8^+$ than $CD4^+$ T_{TN} cells. We also quantified the TREC content of naive T cells from wildlings, a special mouse model which immune phenotype is more similar to that of humans, as it has been suggested that the level of pathogen exposure can have a direct influence on naive T cell dynamics. It appears that naive T cells in wildlings are, just like in SPF mice, mostly produced by the thymus. These findings indicate that the maintenance of naive T cells during homeostasis is differently regulated in mice and men.

Layman's Summary

De thymus is een orgaan dat tijdens de eerste levensjaren van de mens een belangrijke rol speelt in de productie van specifieke afweercellen. Deze cellen worden ook wel naïeve T-cellen genoemd. Naïeve T-cellen kunnen nieuwe ziekteverwekkers die het lichaam binnenkomen herkennen. Daarnaast zorgen naïeve T-cellen ervoor dat ziekteverwekkers weer uit het lichaam verdwijnen. In mensen worden naïeve T-cellen op twee manieren gemaakt: door de thymus en door celdeling. Het is niet bekend *in welke mate* de thymus bijdraagt aan de totale productie van nieuwe naïeve T-cellen. Deze kennis is belangrijk om patiënten met minder naïeve T-cellen beter te kunnen behandelen. Voorbeelden van patiënten met minder naïeve T-cellen zijn mensen waarin op jonge leeftijd de thymus verwijderd is of mensen met een HIV-infectie.

In dit onderzoek bepalen wij het aandeel van de thymus in de productie van naïeve T-cellen in de mens. Dit doen we door het meten van TRECs. TRECs zijn stukjes DNA die vrijkomen in naïeve T-cellen tijdens de productie van deze T-cellen in de thymus. Productie van naïeve T-cellen door de thymus verhoogt daardoor de TREC-inhoud per naïeve T-cel. Hier staat tegenover dat celdeling ervoor zorgt dat de TREC-inhoud per naïeve T-cel daalt. Ons onderzoek laat zien dat de TREC-inhoud per naïeve T-cel vermindert als de mens ouder wordt. Dit geeft aan dat naïeve T-cellen in de mens voornamelijk worden gemaakt door celdeling. Er is maar een erg kleine rol weggelegd voor de thymus in de productie van naïeve T-cellen.

We hebben ook de bijdrage van de thymus aan de productie van naïeve T-cellen in de muis bepaald. Net als voor de mens hebben we dit gedaan met behulp van TREC-metingen. Wetenschappelijk onderzoek wordt vaak uitgevoerd in schone muizen, die weinig tot nooit worden blootgesteld aan ziekteverwekkers. Dit is echter geen goede weergave van de situatie voor de mens. Mensen worden namelijk continu blootgesteld aan ziekteverwekkers. Daarom gebruiken wij voor ons onderzoek een speciale muis, namelijk een wildling. Een wildling-muis is een vieze muis. Door wildling-muizen te gebruiken proberen we het verschil tussen de mens en de muis zo klein mogelijk te maken. Hierdoor verkleinen we de kans dat de overeenkomsten en verschillen die we zien veroorzaakt worden door het diermodel dat we gebruiken. Het blijkt dat de TREC-inhoud per naïeve T-cel niet verandert als de muis ouder wordt. Dit geeft aan dat naïeve T-cellen in wildling-muizen voornamelijk worden gemaakt door de thymus.

Ons onderzoek laat dus zien dat naïeve T-cellen in muizen op een andere manier worden geproduceerd dan in mensen. Muizen worden vaak als model gebruikt om processen in het menselijk lichaam beter te begrijpen. Het is daarom belangrijk om de verschillen in het afweersysteem van de mens en de muis goed te begrijpen. Daarnaast is kennis over de manier waarop naïeve T-cellen worden gemaakt in een gezonde situatie belangrijk om dit proces te begrijpen in patiënten met minder naïeve T-cellen. Hierdoor kunnen we deze patiënten uiteindelijk een betere behandeling aanbieden.

Introduction

The thymus is a primary lymphoid organ responsible for the development of naive T cells. In early childhood, when the thymus starts to involute and active thymus tissue is converted to fat, the contribution of thymopoiesis to naive T cell maintenance rapidly declines[1,2]. Although it can be expected that thymic involution results in a decrease in naive T cell numbers, they remain relatively stable during life[3,4]. It has been proposed that low naive T cell numbers leads to an increase in proliferation[5], thereby keeping the naive $CD4^+$ T cell pool constant[6,7]. Indeed, in healthy human adults, 90% of the naive $CD4^+$ T cell pool is maintained through peripheral proliferation[8]. The remaining 10% is produced by the thymus. One can imagine that lymphopenia, for example following thymectomy early in life, can have a substantial influence on the way naive T cells are produced. Studying the maintenance of naive T cells during healthy aging therefore remains important to further elucidate the effects of lymphopenic conditions on naive T cell dynamics.

The contribution of thymic output to naive T cell maintenance can be quantified through the measurement of T cell receptor excision circles (TRECs)[8]. TRECs are episomal DNA circles, formed during rearrangement of T cell receptor (TCR) genes in both human and murine αβ T cells[9–11]. TRECs are stable[12] and do not replicate during cell division[11]. The observation that TRECs are absent in $CD4^+$ and $CD8^+$ T cells from children with congenital thymic aplasia implies that they are exclusively from thymic origin[11]. The number of $TREC^+$ naive T cells thus represents the number of naive T cells that were produced by the thymus, while the number of $TREC^-$ naive T cells represents the number of naive T cells that arose following peripheral division[8]. Although the TREC content of human single positive (SP) thymocytes remains constant throughout life[8,13], the TREC content of naive T cells declines with age[8,11]. Because TRECs are not replicated during cell division, this explains the decline in TREC content throughout healthy human aging[2,6,10].

Although the compensatory increase in proliferation is a valid explanation for the observed decrease in TREC content, it cannot be excluded that the decline is caused by a bias in the naive T cell population that arises during healthy human aging. Previously, when the TREC content of naive T cells in healthy human individuals was measured, the naive T cell population was selected based on either $CD45RA^+$ or $CD45RO^-CD27^+$ expression[6,8,11]. However, a distinct T cell subset, called stem cell-like memory T cells (T_{SCM} cells), exists within this naive T cell compartment[14]. Phenotypically, T_{SCM} cells are similar to *truly* naive T cells (T_{TN} cells), with either cell type expressing the cell surface markers CD45RA, CD27, CCR7, and CD62L[14]. One way to distinguish T_{SCM} cells from T_{TN} cells is based on the expression of CD95, a cell surface marker that is present on T_{SCM} cells but absent on T_{TN} cells[14]. The percentage of naive $CD4^+$ T cells expressing CD95 only slightly increases with age[15], whereas the percentage of $CD95^+$ cells within the naive $CD8^+$ T cell population reaches almost 20%[15]. Relative to T_{TN} cells, the TREC content of T_{SCM} cells is low[14]. Inclusion of T_{SCM} cells in the naive T cell compartment could hence result in an underestimation of the TREC content, increasing with age due to an expanding T_{SCM} population. To accurately describe the change in TREC content of naive T cells with age, the TREC content of T_{TN} cells should therefore be quantified. The hypothesis is that the TREC content of T_{TN} cells reduces with age, but in lesser extent to what has previously been shown in naive T cells.

In contrast to what has been observed in humans, the TREC content of murine naive T cells does not significantly decrease with age[8], suggesting that the peripheral naive T cell pool in mice is mostly

maintained through thymic output. It thus appears that the maintenance of the naive T cell pool in mice and humans is differentially regulated. However, the data collected on murine naive T cell dynamics comes from experiments done in specific pathogen-free (SPF) C57BL/6 mice[8]. While the immune system of SPF mice is relatively antigen-inexperienced, the human immune system is constantly exposed to pathogens[16]. It has already been demonstrated in mice that the level of antigen exposure can have a direct effect on naive T cell dynamics[17]. Housing in an antigen-rich environment results in a more rapid involution of the thymus[17]. Unsurprisingly, dirty mice show a much faster decline in naive T cell numbers throughout their life compared to mice that are housed in clean facilities[17]. To further elucidate the influence of an antigen-experienced immune state on naive T cell dynamics, a new mouse model, termed *wildling*, has been developed[18]. The skin, gut, and vaginal microbiota, and splenic immune phenotype of wildlings more closely resembles the microbiota and splenic immune phenotype of wild mice than that of SPF mice[18]. Quantifying the TREC content of naive T cells in wildlings will provide more inside on the influence of an antigen-rich environment on naive T cell dynamics and whether the way the naive T cell pool is maintained, is species-specific.

In this project, the maintenance of the naive T cell pool in mice and humans has been re-evaluated. The contribution of the thymus to the homeostatic maintenance of the naive T cell compartment was quantified by measuring the TREC content of T_{TN} cells from humans and naive T cells from wildlings. Our data confirm that the human truly naive T cell compartment is almost exclusively maintained through peripheral division. Remarkably, the thymus plays a more prominent role in the maintenance of $CD8^+$ than $CD4^+$ T_{TN} cells. We also show that the naive T cell population in wildlings is mostly produced by the thymus, suggesting that the homeostatic maintenance of naive T cells is truly differently regulated in mice and men. As mice are a common model used to study the effects of healthy human aging[2] and thymectomy[19] on naive T cell dynamics, understanding this difference is of great importance.

Material & Methods

Human participants

Five umbilical cord blood samples were obtained from healthy full-term newborns and the peripheral blood mononuclear cell (PBMC) fractions were stored in liquid nitrogen in the Universitair Medisch Centrum Utrecht (UMCU) Biobank until further use. Venous blood samples from thirty-one healthy human adults were collected, either through the Mini Donor Dienst at the UMCU *(N=21)* or from individuals that participated in the DICE study (METC 15/745) *(N=10)*. The DICE study was approved by the local ethical committee of the UMCU and conducted in accordance with the Helsinki declaration, last amended in 2013.

Mice

Female C57BL/6 wildlings (*N=49*), kindly provided to us by Stephan Rosshart, were housed at the Poonawalla Sciencie Park B.V. in Bilthoven, The Netherlands. The wildlings ranged from ten to twenty-nine weeks in age and were housed in Makrolon type 2L cages containing both bedding and nesting material, shelter, and an exercise wheel. All animal experiments were approved by the Animal Experiments Committee Utrecht.

Cell preparation

Human peripheral blood was collected in sodium heparin tubes, after which it was diluted 1:1 with PBS (Sigma-Aldrich). PBMCs were obtained through Ficoll-Paque PLUS density gradient centrifugation. Following isolation, PBMCs were viably frozen in Nunc™ Cryotube™ vials (Thermo Fisher Scientific), containing 90% FBS and 10% DMSO (Sigma-Aldrich), at -80°C. Single cell suspensions of murine thymocytes and splenocytes were obtained by mechanical disruption of the thymus and spleen. Erythrocytes in spleen samples were lysed with 1X Red Blood Cell lysis buffer, containing 155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.13 mM EDTA.

Fluorescence-activated Cell Sorting (FACS)

Frozen human PBMC samples were thawed using RPMI medium (Thermo Fisher Scientific) supplemented with 20% FBS. PBMCs were stained with CD3-AF700, CD8a-PerCP-Cy5.5, CD4-BV785, CD45RO-BV711, CCR7-PE-Cy7 (Biolegend), CD27-BV510 and CD95-APC (BD Biosciences) in order to sort T_{TN} cells ($CD45RO^-CD27^+CCR7^+CD95^-$), $CD95^+$ naive T cells ($CD45RO^-CD27^+CCR7^-CD95^+$) and central memory T cells (T_{CM} cells) ($CD45RO^+CD27^+$). The complete gating strategy is depicted in Supplementary Figure 1. Previously, PBMCs from the DICE participants were stained with CD3-PerCP, CD8a-BV510, CCR7-BV421, CD45RO-PE-Cy7 (Biolegend), CD27-APC-R700 (BD Biosciences), CD4-APC-eF780 and CD95-FITC (eBioscience), and sorted for T_{TN} and T_{CM} cells. Fresh splenocytes from wildlings were treated with Fc-Block (BD Biosciences) and stained with CD45-APC-eF780 (eBioscience), CD3e-PE-Cy5, CD44-BV605 (Biolegend), CD8a-BV786, CD4-AF700, CD62L-BV650 and CD69-PE-Cy7 (BD Biosciences) in order to obtain naive T cells ($CD62L^+CD44^-$) and $CD69^-$ memory T cells ($CD44^+CD69^-$). The full gating strategy can be found in Supplementary Figure 2. Murine thymocytes were stained with CD45-APC-eF780 (eBioscience), CD3-FITC, CD8a-BV421 and CD4-PE-CF594 (BD Biosciences), and sorted for SP $CD4^+$ and $CD8^+$ thymocytes. The BD FACSAria™ III Cell Sorter was used for sorting and data was analyzed with BD FACSDiva™ V8 and FlowJo V10 software. DNA of the sorted fractions was isolated using the ReliaPrep™ Blood gDNA Miniprep System (Promega), with the elution volume being adjusted based on the amount of sorted cells: 100 µL for samples >300000 cells and 70 µL for samples <300000 cells.

Human TREC assay

The TREC content of human T_{TN} cells, $CD95^+$ naive T cells and T_{CM} cells was quantified using qPCR on the Quantstudio 12K Flex System. The sj TREC primer and probe sequences were as follows: forward primer 5'-CCATGCTGACACCTCTGGTT-3', reverse primer 5'-TCGTGAGAACGGTGAATGAAG-3' and probe 5'-/56-FAM/CACGGTGATGCATAGGCACCTGC/36-TAMsp/-3'. Each sj TREC qPCR sample contained 1X Taqman™ Fast Advanced MasterMix (Thermo Fisher Scientific), 1.33 µM forward and reverse primer, 0.67 µM probe and 5 µL of isolated DNA. Based on a standard curve that was created with a sj TREC plasmid, consisting of a pUC19 vector cloned with the sj TREC fragment at the HindIII restriction site, the amount of sj TREC copies could be determined for each qPCR sample. More information on the sj TREC plasmid can be found in the Supplementary Methods and Supplementary Figure 3. To compensate for input DNA, the Cα gene was used as a reference: forward primer 5'-CCTGATCCTCTTGTCCCACAG-3', reverse primer 5'-GGATTTAGAGTCTCTCAGCTGGTACA-3' and probe 5'-/56-FAM/ATCCAGAACCCTGACCCTGCCG/36-TAMsp/-3'. Each Cα qPCR sample contained 1X Taqman™ Fast Advanced MasterMix (Thermo Fisher Scientific), 1.33 µM forward and reverse primer, 0.33 µM probe and 5 µL of isolated DNA. The following qPCR program was used to amplify the DNA: 2min. 50°C, 10min. 95°C, 15sec. 95°C and 1min. 60°C, for 40 cycles. The amount of sj TREC copies/ng DNA was calculated by dividing the median sj TREC copy number by the median quantity (ng) of Cα DNA. Based on the assumption that 150000 cells together contain 1 µg of DNA[20,21], the TREC content of human T_{TN} cells, $CD95^+$ naive T cells and T_{CM} cells was quantified by dividing the amount of sj TREC copies/ng DNA by 150. qPCR samples that generated a Ct value >36 were excluded from analysis.

Murine TREC assay

The TREC content of murine SP thymocytes and naive T cells from the spleen was also quantified using qPCR on the Quantstudio 12K Flex System. The sj TREC primer and probe sequences were as follows: forward primer 5'-CCAAGCTGACGGCAGGTTT-3', reverse primer 5'-AGCATGGCAAGCAGCACC-3' and probe 5'-/56-FAM/TGCTGTGTGCCCTGCCCTGCC/36-TAMsp/-3'. To compensate for input DNA, the CD45 gene was used as a reference: forward primer 5'-TCAGAGGCCAGGCTCACTCAAG-3', reverse primer 5'-CTAGGCCAACCACTCCCACTGT-3' and probe 5'-/56-FAM/CAAGTTGCCCAGCGATGCCAGC/36-TAMsp/-3'. The sj TREC as well as the CD45 qPCR samples contained 1.0x Taqman™ Fast Advanced MasterMix (Thermo Fisher Scientific), 300 nM forward and reverse primer, 200 nM probe and 5 µL of isolated DNA. The following qPCR program was used to amplify the DNA: 2min. 50°C, 10min. 95°C, 15sec. 95°C and 1min. 60°C, for 40 cycles. The TREC content of murine thymocytes and naive T cells was calculated by normalizing the sj TREC standard curve to the CD45 standard curve[9], with the assumption that 150000 cells together contain 1 µg of DNA and each cell carries two CD45 copies (Equation 1)[9].

$$TREC\ content = (1 + E)^{-(\Delta Ct)}\ x\ 2 \qquad \text{(Equation 1)}$$

E = qPCR efficiency

ΔCt = Ct $Y_{intercept}$ sj TREC standard curve - Ct $Y_{intercept}$ CD45 standard curve

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.3 software. Differences between paired groups were evaluated using the Wilcoxon signed-rank test. Correlations were assessed with the Spearman rank-order correlation coefficient. p-values < 0.05 were considered significant.

Results

The human thymus contributes more to the maintenance of CD8⁺ than CD4⁺ T_{TN} cells

In order to determine the relative contribution of the thymus to the maintenance of the human naive T cell compartment, we quantified the TREC content of CD4⁺ and CD8⁺ T_{TN} cells from healthy human donors. The TREC content of both CD4⁺ and CD8⁺ T_{TN} cells remained relatively constant for the first twenty years of life, after which the TREC content started to decline (Figure 1A). The TREC content of both CD4⁺ and CD8⁺ T_{TN} cells declined significantly with age (CD4⁺ T_{TN} cells: $p < 0.0001$ and $r_s = -0.6979$, CD8⁺ T_{TN} cells: $p = 0.0058$ and $r_s = -0.6076$). But where the TREC content of CD4⁺ T_{TN} cells declined ~50 times throughout life, the TREC content of CD8⁺ T_{TN} cells only decreased ~3.4 fold. Further analysis revealed that the TREC content of CD8⁺ T_{TN} cells was higher than the TREC content of CD4⁺ T_{TN} cells in almost every donor (Figure 1B). Moreover, the median TREC content of CD8⁺ T_{TN} cells turned out to be significantly higher compared to the median TREC content of CD4⁺ T_{TN} cells ($p = 0.0001$). Because not every naive T cell that exits the thymus carries a TREC[8], the TREC content of CD4⁺ and CD8⁺ T_{TN} cells has to be normalized to the TREC content of recent thymic emigrants (RTEs) in order to quantify the relative contribution of the thymus to naive T cell maintenance. We used the average TREC content of CD4⁺ and CD8⁺ T_{TN} cells from umbilical cord blood samples as an estimation for the TREC content of CD4⁺ and CD8⁺ RTEs respectively. On average, ~22% of the CD4⁺ T_{TN} pool in adults had been formed by the thymus, while this was ~36% in the case of CD8⁺ T_{TN} cells (Figure 1C). This means that ~78% of the CD4⁺ and ~64% of the CD8⁺ T_{TN} cell compartment had been maintained through peripheral division.

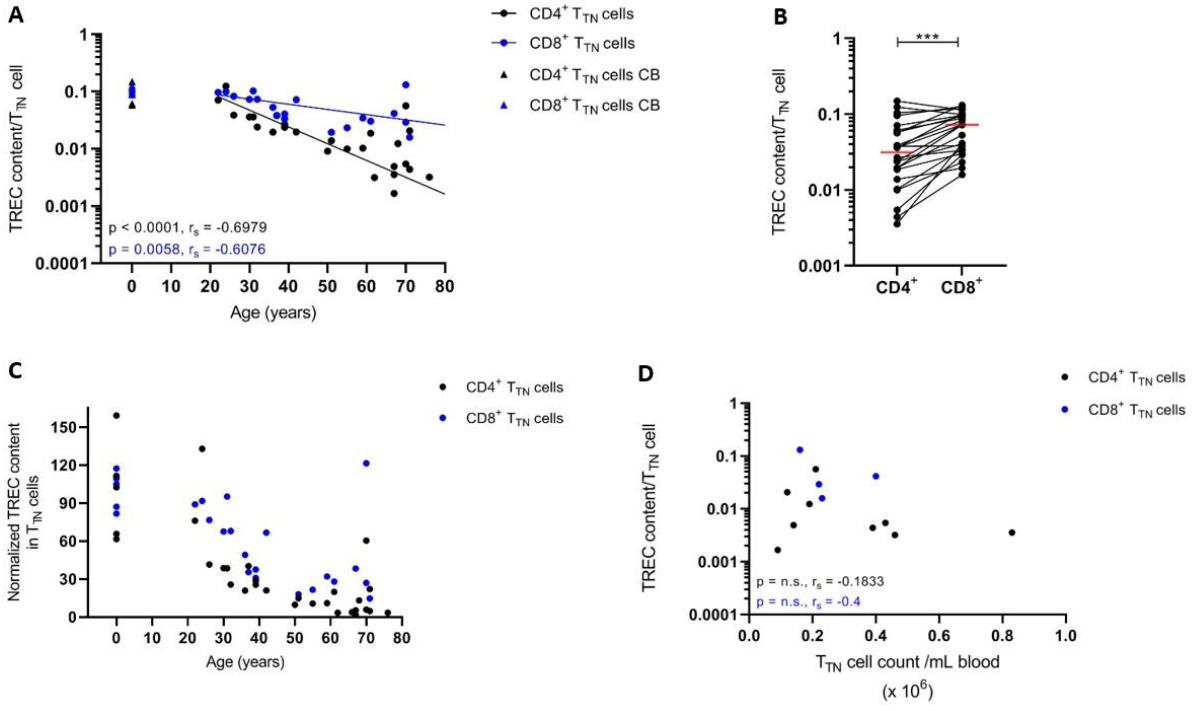


Figure 1: The human thymus contributes more to the maintenance of CD8⁺ than CD4⁺ T_{TN} cells. A) The TREC content per cell is shown for sorted human CD4⁺ (black dots) and CD8⁺ T_{TN} cells (blue dots) from peripheral blood *(N = 31)* and umbilical cord blood *(CB, N = 5)*. B) The median TREC content of paired CD4⁺ and CD8⁺ T_{TN} cells (red horizontal lines). C) Normalized TREC content in human CD4⁺ (black dots) and CD8⁺ T_{TN} cells (blue dots), calculated by dividing the TREC content of CD4⁺/CD8⁺ T_{TN} cells by the average TREC content of CD4⁺/CD8⁺ T_{TN} cells from umbilical cord blood samples. D) The TREC content of human CD4⁺ (black dots) and CD8⁺ T_{TN} cells (blue dots) as a function of the T_{TN} cell count/mL blood. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (Spearman's correlation test (a,d), Wilcoxon signed-rank test (b)).

These data suggest that the human thymus is somewhat more involved in the production of $CD8^+$ than $CD4^+$ T_{TN} cells. The spread in TREC content/T_{TN} cell between the older individuals (> 65 years of age) turned out to be relatively large in comparison to the other age groups. With a similar production rate of $TREC^+$ T_{TN} cells, but a higher amount of T_{TN} cells/mL blood, the TREC content/T_{TN} cell goes down. To exclude the possibility that the observed decline in TREC content/T_{TN} cell is confounded by the amount of T_{TN} cells/mL blood, we looked at the relationship between these two values (Figure 1D). For both $CD4^+$ and $CD8^+$ T_{TN} cells, no clear inverse correlation appeared to be present between the TREC content/T_{TN} cell and the T_{TN} cell count/mL blood ($CD4^+$ T_{TN} cells: p = n.s and r_s = -0.1833, $CD8^+$ T_{TN} cells: p = n.s. and r_s = -0.4). We also quantified the TREC content of $CD4^+$ and $CD8^+$ T_{CM} cells (Supplementary Figure 4). In accordance with previous research[11,14,22], the TREC content of T_{CM} cells was lower compared to the TREC content of T_{TN} cells. The transition from a naive to memory phenotype is accompanied with many rounds of cell division[11,22]. As TRECs are not replicated during cell division[11], this explains the observed difference in TREC content. Using the normalized TREC content and daily production rate of T_{TN} cells, we calculated the daily thymic output using the following equation[8]:

$$\frac{A}{c} = \frac{\sigma(t)}{\sigma(t)+pN(t)} \qquad \text{(Equation 2)}$$

with A being the TREC content of T_{TN} cells, c the average TREC content of T_{TN} cells from umbilical cord blood samples, $\sigma(t)$ the thymic output of T_{TN} cells at time t, p the proliferation rate of T_{TN} cells and $N(t)$ the total amount of T_{TN} cells at time t. For ten donors, the normalized TREC content $\frac{A}{c}$ and the daily thymic output $\sigma(t)$ are shown in Table 1, while the proliferation rate p and total amount of cells $N(t)$ are depicted in Supplementary Table 1. The median thymic output of $CD4^+$ and $CD8^+$ T_{TN} cells turned out to be $0.40x10^7$ and $1.52x10^7$ cells/day respectively. The median total amount of $CD4^+$ T_{TN} cells in the body was $5.11x10^{10}$, meaning that the thymus produces 0.0078% of the total amount of $CD4^+$ T_{TN} cells on a daily basis. The median total amount of $CD8^+$ T_{TN} cells turned out to be $1.84x10^{10}$, which means that the thymus produces 0.083% of the total amount of $CD8^+$ T_{TN} cells per day. These data strengthen our hypothesis that the human thymus is more involed in the maintenance of $CD8^+$ than $CD4^+$ T_{TN} cells.

Exclusion of $CD95^+$ naive T cells does not influence the decline in TREC content during healthy human aging

Previously, we also observed a decline in TREC content of human naive T cells with age[6,8]. However, we measured the TREC content of the whole human naive T cell compartment, in which T_{SCM} cells are still included, rather than of *truly* naive T cells. Where *conventional* naive T cells were previously selected based on $CD45RO^-CD27^+$ expression[6,8] (Figure 2A), we now selected *truly* naive T cells based on $CD45RO^-CD27^+CCR7^+CD95^-$ expression (Figure 2B). It is good to emphasize that the population we excluded, $CD95^+$ naive T cells ($CD45RO^-CD27^+CCR7^-CD95^+$), did not consist purely of T_{SCM} cells, as T_{SCM} cells express both CCR7 and CD95. For three donors, we also measured the TREC content of $CD4^+$ and $CD8^+$ $CD95^+$ naive T cells (Figure 2C). However, in 5 of the 6 $CD95^+$ naive T cell samples, the amount of sj TREC DNA was so little that no reliable TREC content was retrieved. The TREC content of the remaining $CD4^+$ $CD95^+$ naive T cell sample turned out to be lower compared to TREC content of the paired $CD4^+$ T_{TN} cell sample. Thus even though we did not we did not filter out pure T_{SCM} cells, we did exclude a population that has a lower TREC content compared to T_{TN} cells. We also confirmed that the

Donor	Age (years)	Normalized TREC content **CD4⁺** T_{TN} cells	Total daily production **CD4⁺** T_{TN} cells ($x10^7$) (cells/day)	Daily thymic output **CD4⁺** T_{TN} cells ($x10^7$) (cells/day)	Normalized TREC content **CD8⁺** T_{TN} cells	Total daily production **CD8⁺** T_{TN} cells ($x10^7$) (cells/day)	Daily thymic output **CD8⁺** T_{TN} cells ($x10^7$) (cells/day)
DICE E04	70	60%	0.93	0.56	121%	0.47	0.57
DICE E08	67	5%	6.23	0.31	N.D.	2.01	N.D.
DICE E10	71	5%	13.34	0.67	15%	7.87	1.18
DICE E21	70	N.D.	3.35	N.D.	N.D.	1.18	N.D.
DICE E23	71	22%	1.82	0.40	N.D.	0.28	N.D.
DICE E30	67	4%	34.48	1.38	38%	9.59	3.64
DICE E34	68	13%	2.07	0.27	N.D.	0.55	N.D.
DICE E35	70	6%	19.36	1.16	27%	6.90	1.86
DICE E39	67	2%	0.65	0.01	N.D.	0.02	N.D.
DICE E59	76	3%	1.21	0.04	N.D.	0.47	N.D.
Median		5%	2.71	0.40	32.5%	0.86	1.52

Table 1: Daily thymic output of human $CD4^+$ and $CD8^+$ T_{TN} cells. The normalized TREC content of human $CD4^+$ and $CD8^+$ T_{TN} cells was calculated by dividing the TREC content of $CD4^+/CD8^+$ T_{TN} cells by the average TREC content of $CD4^+/CD8^+$ T_{TN} cells from umbilical cord blood samples. The total daily production of $CD4^+$ and $CD8^+$ T_{TN} cells was calculated as follows: p (proliferation rate of $CD4^+/CD8^+$ T_{TN} cells (Van den Berg *et al.* (submitted)) x (amount of $CD4^+/CD8^+$ T_{TN} cells per 5L whole blood (Van den Berg *et al.* (submitted)) x 50 (assuming that only 2% of the lymphocytes reside in the blood[8]). The proliferation rate p of T_{TN} cells was estimated through ^2H_2O labeling (Van den Berg *et al.* (submitted)). The total daily production represents the sum of the daily thymic output of $CD4^+/CD8^+$ T_{TN} cells and the production of $CD4^+/CD8^+$ T_{TN} cells through peripheral division. The daily thymic output of $CD4^+$ and $CD8^+$ T_{TN} cells was calculated by multiplying the normalized TREC content of $CD4^+/CD8^+$ T_{TN} cells with the total daily production of $CD4^+/CD8^+$ T_{TN} cells. N.D. = not determined.

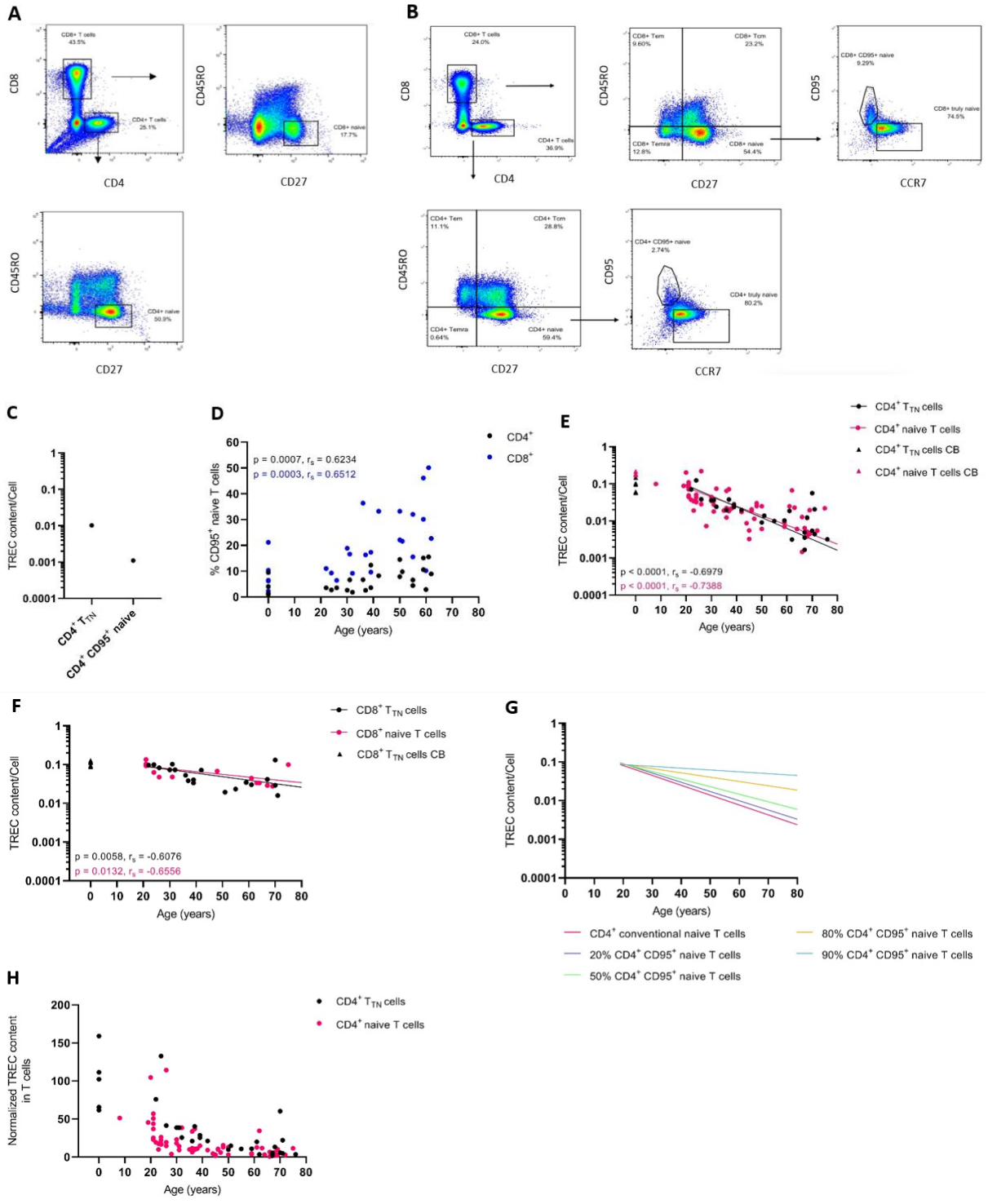


Figure 2: Exclusion of CD95⁺ naive T cells does not influence the decline in TREC content during healthy human aging. A) The sorting strategy that was previously used for human CD4⁺ and CD8⁺ conventional naive T cells (CD45RO⁻CD27⁺) is shown. B) The sorting strategy for human CD4⁺ and CD8⁺ truly naive T cells (CD45RO⁻CD27⁺CCR7⁺CD95⁻). C) The TREC content per cell is depicted for paired CD4⁺ T_{TN} cells and CD4⁺ CD95⁺ naive T cells. D) The percentage CD4⁺ (black dots) and CD8⁺ (blue dots) CD95⁺ naive T cells as a function of age. E) The TREC content per cell is shown for human CD4⁺ T_{TN} cells (black dots) and CD4⁺ conventional naive T cells (pink dots). F) The TREC content per cell for human CD8⁺ T_{TN} cells (black dots) and CD8⁺ conventional naive T cells (pink dots). The data on the TREC content of CD4⁺ and CD8⁺ conventional naive T cells was retrieved from two independent studies. G) Model of the TREC content per cell considering different percentages of CD4⁺ CD95⁺ naive T cells within the CD4⁺ conventional naive T cell compartment. This model was created by taking the TREC content of CD4⁺ conventional naive T cells (pink line) as the basis. It was assumed that no CD4⁺ CD95⁺ naive T cells were excluded from the conventional naive T cells population. Consequently, the decline in TREC content per T_{TN} cell was modelled, considering that

the increase in $CD4^+$ $CD95^+$ naive T cells during healthy human aging was either 20% (dark blue line), 50% (green line), 80% (yellow line) or 90% (light blue line). The TREC content of $CD4^+$ $CD95^+$ naive T cells was set at zero. H) Normalized TREC content in human $CD4^+$ T_{TN} cells (black dots) and $CD4^+$ conventional naive T cells (pink dots), calculated by dividing the TREC content of $CD4^+$ T_{TN} cells and $CD4^+$ conventional naive T cells by the average TREC content of $CD4^+$ T_{TN} cells and $CD4^+$ conventional naive T cells from umbilical cord blood samples. CB = cord blood. (Spearman's correlation test (d,e,f)).

percentage $CD95^+$ naive T cells significantly increases during healthy human aging ($CD4^+$ $CD95^+$ naive T cells: $p = 0.0007$ and $r_s = 0.6234$, $CD8^+$ $CD95^+$ naive T cells: $p = 0.0003$ and $r_s = 0.6512$), with the $CD8^+$ $CD95^+$ naive T cell compartment expanding at a higher rate than the proportion of $CD4^+$ $CD95^+$ naive T cells (Figure 3D). On average, healthy human adults contained 7.2% $CD4^+$ $CD95^+$ naive T cells, with a range from 2.74% (lowest % in donors < 30 years) to 15.5% (highest % in donors > 55 years). In the case of $CD8^+$ $CD95^+$ naive T cells, the average percentage was 22.3%, with a range from 6.41% to 50.1%. Next, we assessed whether the decline in TREC content of T_{TN} cells differed from that of conventional naive T cells (Figure 3E,F). The data on the TREC content of conventional naive T cells was retrieved from two independent studies. The TREC content of T_{TN} cells as well as conventional naive T cells decreased significantly with age ($CD4^+$ T_{TN} cells: $p < 0.0001$ and $r_s = -0.6979$, $CD4^+$ conventional naive T cells: $p < 0.0001$ and $r_s = -0.7388$, $CD8^+$ T_{TN} cells: $p = 0.0058$ and $r_s = -0.6076$, $CD8^+$ conventional naive T cells: $p < 0.0132$ and $r_s = -0.6556$). Both in the case of $CD4^+$ and $CD8^+$, the slopes of the two regression lines did not differ significantly ($CD4^+$: $p = 0.8170$, $CD8^+$: $p = 0.7130$). These data show that exclusion of the $CD95^+$ naive T cell population does not influence the decline in TREC content during healthy human aging. We would not have expected this based on our findings that $CD95^+$ naive T cells, which increase in proportion throughout life, have a relatively low TREC content compared to T_{TN} cells. We hypothesized that the lack of difference between the decline in TREC content of T_{TN} cells and conventional naive T cells could be due to the relative low percentage of $CD95^+$ naive T cells that was excluded. We therefore modeled what the increase in percentage of $CD4^+$ $CD95^+$ naive T cells during healthy human aging should have been in order to detect a signifcant difference between the decline in TREC content of T_{TN} cells and conventional naive T cells (Figure 2G). We took the TREC content of $CD4^+$ conventional naive T cells as the basis and assumed that no $CD4^+$ $CD95^+$ naive T cells were excluded from that population. We then modelled the TREC content of T_{TN} cells, assuming that the increase of $CD4^+$ $CD95^+$ naive T cells during healthy human aging reached either 20%, 50%, 80% or 90% and that these cells were excluded. We set the TREC content of $CD4^+$ $CD95^+$ naive T cells at zero. Suprisingly, even the slope of the regression line considering a 90% increase in $CD4^+$ $CD95^+$ naive T cells did not differ significantly from the slope of the regression line from conventional naive T cells ($p = 0.1877$). This indeed suggests that the percentage of $CD4^+$ $CD95^+$ naive T cells that we excluded was too small to see a significant difference between the decline in TREC content of T_{TN} and conventional naive T cells. To confirm that the human truly naive T cell compartment is almost exclusively maintained through peripheral division, we also compared the normalized TREC content in $CD4^+$ T_{TN} cells to the normalized TREC content in $CD4^+$ conventional naive T cells (Figure 2H). On average, ~18% of the $CD4^+$ conventional naive T cell compartment had been produced by the thymus, meaning that ~82% was formed through peripheral division. This does not differ much from the percentage of T_{TN} cells that had been formed by peripheral proliferation, which was already determined to be ~78%. We hereby confirm that human *truly* naive T cells are mainly produced through peripheral division.

Naive T cells in wildlings are mainly produced through thymic output

Our findings thus confirmed that the human naive T cell compartment is mainly maintained through peripheral division. To evaluate whether the way the naive T cell pool is maintained during homeostasis is species-specific, we also quantified the TREC content of naive T cells from mice. As it has been proposed that the level of antigen exposure can directly influence naive T cell dynamics[17], we used wildling mice instead of SPF mice as our mouse model. We started by looking for an effect of age on the thymus tissue of wildlings (Figure 3A). It turned out that an inverse correlation exists between age and thymus weight ($p < 0.0001$ and $r_s = -0.5952$), indicating that the thymus of wildlings involutes throughout their life. Next, we quantified the TREC content of SP $CD4^+$ and $CD8^+$ thymocytes as well as $CD4^+$ and $CD8^+$ naive T cells from the spleen (Figure 3B). Remarkably, the TREC content of both $CD4^+$ and $CD8^+$ naive T cells significantly increased between ten and twenty-nine weeks of age ($CD4^+$ naive T cells: $p < 0.0001$ and $r_s = 0.7055$, $CD8^+$ naive T cells: $p < 0.0001$ and $r_s = 0.6426$). In four wildlings, we were also able to measure the TREC content of $CD4^+$ and $CD8^+$ $CD69^-$ memory T cells (Figure 3B). As expected, the TREC content of memory T cells turned out to be lower than the TREC content of their naive counterparts. Further analysis revealed that, opposite to what we observed in humans, the median TREC content of murine $CD8^+$ naive T cells was significantly lower in comparison to the TREC content $CD4^+$ naive T cells (Figure 3C). In order to quantify the relative contribution of the thymus to murine naive T cell maintenance, we normalized the TREC content of $CD4^+$ and $CD8^+$ naive T cells to the TREC content of SP $CD4^+$ and $CD8^+$ thymocytes respectively (Figure 3D). On average, the thymus

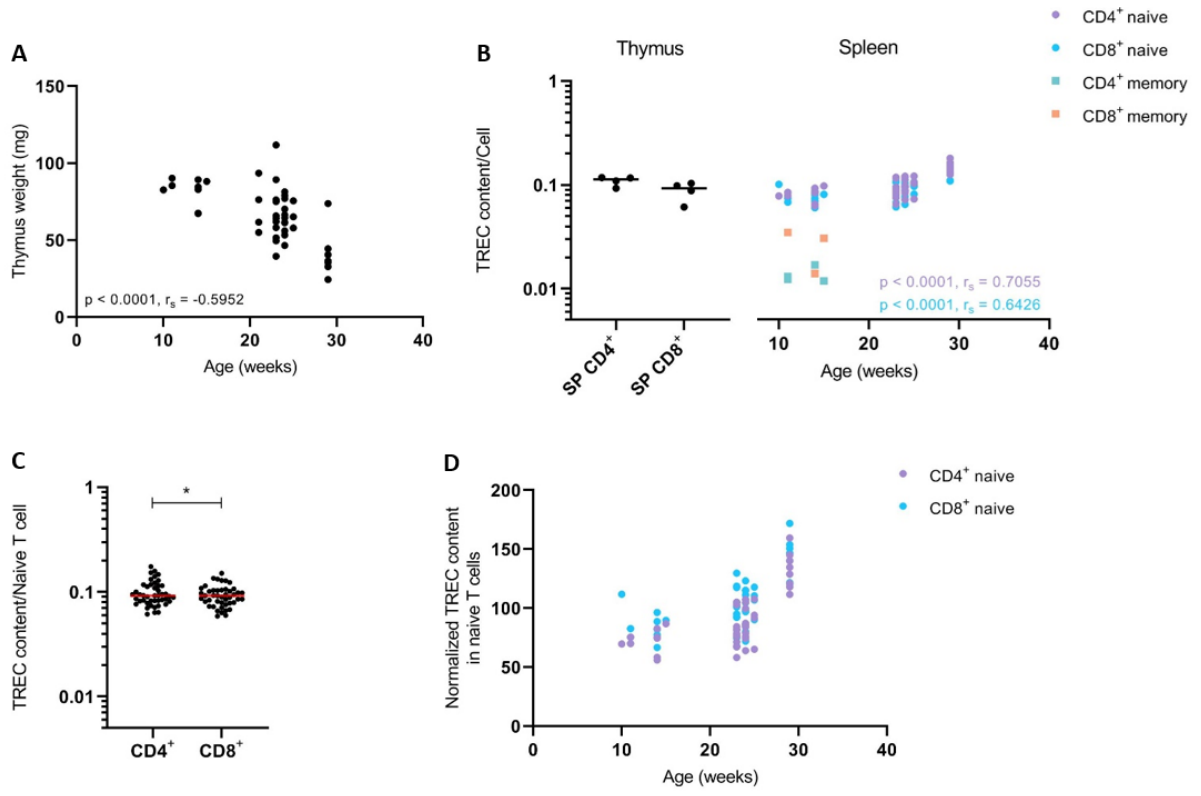


Figure 3: Naive T cells in wildlings are mainly produced through thymic output. A) The thymus weight of wildlings as a function of age. B) The TREC content per cell is shown for murine SP $CD4^+$ and $CD8^+$ thymocytes (black dots), $CD4^+$ (purple dots) and $CD8^+$ (light blue dots) naive T cells as well as $CD4^+$ (green dots) and $CD8^+$ (orange dots) $CD69^-$ memory T cells from the spleen. C) The median TREC content of paired murine $CD4^+$ and $CD8^+$ naive T cells (red horizontal lines). D) Normalized TREC content in murine $CD4^+$ (purle dots) and $CD8^+$ (light blue dots) naive T cells, calculated by dividing the TREC content of murine $CD4^+$/$CD8^+$ naive T cells by the average TREC content of SP $CD4^+$/$CD8^+$ thymocytes respectively. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (Spearman's correlation test (a,b), Wilcoxon signed-rank test (c)).

produced 90% of the $CD4^+$ and 105% of the $CD8^+$ naive T cell pool, implying that the naive T cell compartment in wildlings is almost exclusively maintained through thymic output. It also suggests that the thymus contributes slightly more to the production of $CD8^+$ than $CD4^+$ naive T cells, similar to what we observed in humans.

Discussion

We re-evaluated the maintenance of the naive T cell pool during homeostasis in humans and mice. Instead of looking at the human *conventional* naive T cell compartment ($CD45RO^-CD27^+$), which includes T_{SCM} cells, we focused on the most pure form of human naive T cells, termed *truly* naive T cells ($CD45RO^-CD27^+CCR7^+CD95^-$). We found that for the first twenty years of life, the TREC content of human $CD4^+$ and $CD8^+$ T_{TN} cells remains relatively stable. This indicates that the thymus is able to compensate for the dilution of TRECs amongst T_{TN} cells as a result of peripheral division, in line with the fact that the thymus is very active in early childhood[1], producing sufficient amounts of $TREC^+$ T_{TN} cells[1,2]. From puberty on, when the thymus starts to involute, the relative contribution of the thymus to T_{TN} cell production declines[1,2]. The thymus is hence less able to compensate for the TREC dilution amongst T_{TN} cells following peripheral division, explaining the decline in TREC content after adolescence is reached.

We also show that the TREC content of $CD8^+$ T_{TN} cells declines less over time compared to the that of $CD4^+$ T_{TN} cells. This seemed logical, as it has already been decribed that $CD8^+$ *conventional* naive T cells have a lower proliferation rate in comparison to $CD4^+$ *conventional* naive T cells[19]. However, our data suggest that, at least in older individuals (> 65 years of age), the proliferation rate of $CD4^+$ and $CD8^+$ *truly* naive T cells is comparable. Assuming that the TREC content of SP thymocytes[8,13] as well as the proliferation rate of T_{TN} cells does not change with age[15], a similar proliferation rate results in a similar dilution of TRECs amongst $CD4^+$ and $CD8^+$ T_{TN} cells during healthy human aging. To explain the different decline in TREC content of $CD4^+$ and $CD8^+$ T_{TN} cells we therefore looked at the daily thymic output. We found that the daily thymic output of $CD8^+$ T_{TN} cells (median $1.52x10^7$ cells/day) is higher in comparison to that of $CD4^+$ T_{TN} cells (median $0.40x10^7$ cells/day). As the TREC content of $CD4^+$ and $CD8^+$ T_{TN} cells from ubilical cord blood samples turned out to be comparable, we can assume that the TREC content of $CD4^+$ and $CD8^+$ RTEs is the same. The absolute number of $TREC^+$ $CD8^+$ T_{TN} cells that enter the periphery on a daily basis is thus higher compared to the number of $TREC^+$ $CD4^+$ T_{TN} cells. Although the daily thymic output of $CD4^+$ T_{TN} cells turned out to be lower in comparison to that of $CD8^+$ T_{TN} cells, the total daily production rate of $CD4^+$ T_{TN} cells was higher ($CD4^+$: median $2.71x10^7$ cells/day, $CD8^+$: $0.86x10^7$ cells/day). Considering that the proliferation rate of $CD4^+$ and $CD8^+$ T_{TN} cells is comparable, this suggests that the number of $TREC^-$ $CD4^+$ T_{TN} cells that are produced per day is higher compared to the number of $TREC^-$ $CD8^+$ T_{TN} cells. These two different processes together, of which a schematic overview is depicted in figure 4, explain the faster decline in TREC content of $CD4^+$ than $CD8^+$ T_{TN} cells during healthy human aging.

Because previously, we also observed a decline in TREC content of human *conventional* naive T cells with age[6,8], we compared the TREC content of T_{TN} cells to that of conventional naive T cells. It turned out that, both in the case of $CD4^+$ and $CD8^+$, the decline in TREC content of T_{TN} cells did not differ signficantly from the decline in TREC content of conventional naive T cells. This was unexpected, as we found that $CD95^+$ naive T cells contain a relatively low TREC content compared to T_{TN} cells and increase in proportion with age. We already showed that the lack of difference in TREC content decline is probably due to the relatively low percentage of $CD95^+$ naive T cells that was excluded. This low percentage of $CD95^+$ naive T cells in combination with the relatively tight gate for conventional naive T cells ($CD45RO^-CD27^+$) made us check the purity of the conventional naive T cells. As it turned out, the

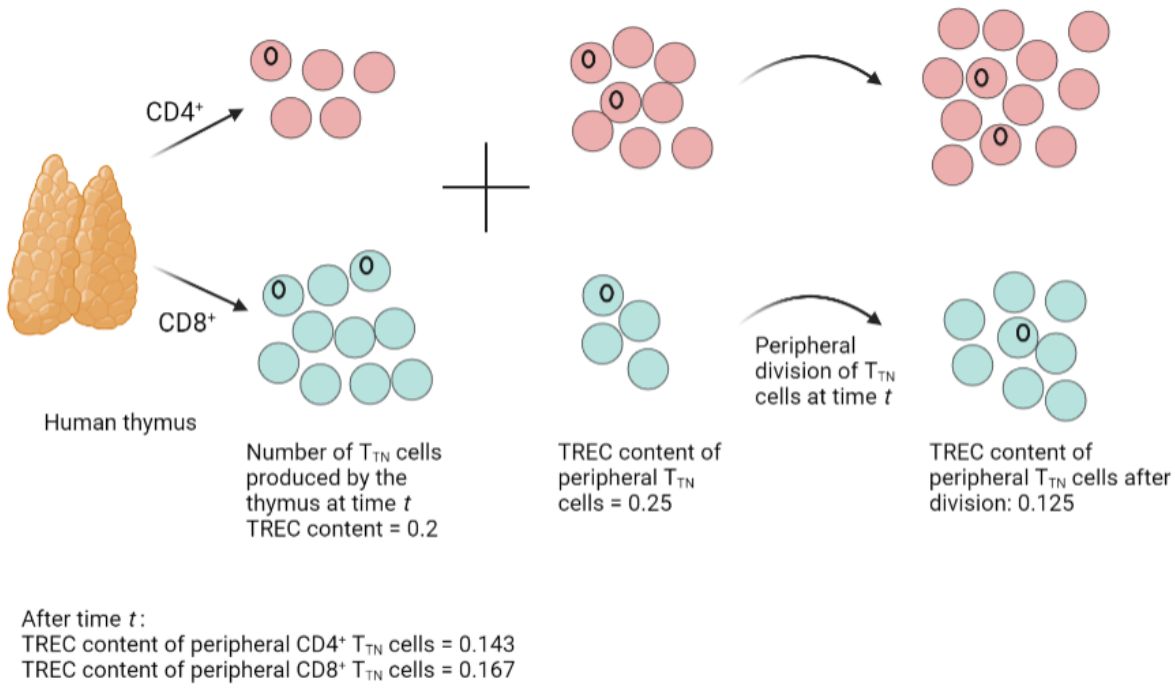


Figure 4: Schematic representation of the production of $CD4^+$ and $CD8^+$ T_{TN} cells through thymic output as well as peripheral division. The daily thymic output of $CD8^+$ T_{TN} cells is higher in comparison to that of $CD4^+$ T_{TN} cells. As the TREC content of $CD4^+$ and $CD8^+$ T_{TN} cells from ubilical cord blood samples turned out to be comparable, we can assume that the TREC content of $CD4^+$ and $CD8^+$ RTEs is the same. The number of $TREC^+$ $CD8^+$ T_{TN} cells that enter the periphery on a daily basis is thus higher compared to the number of $TREC^+$ $CD4^+$ T_{TN} cells. Although the daily thymic output of $CD4^+$ T_{TN} cells turned out to be lower in comparison to that of $CD8^+$ T_{TN} cells, the total daily production rate of $CD4^+$ T_{TN} cells was higher. Considering that the proliferation rate of $CD4^+$ and $CD8^+$ T_{TN} cells is more or less the same, this suggests that the number of $TREC^-$ $CD4^+$ T_{TN} cells that are produced per day is higher compared to the number of $TREC^-$ $CD8^+$ T_{TN} cells. These two different processes together explain the faster decline in TREC content of $CD4^+$ than $CD8^+$ T_{TN} cells during healthy human aging. In this figure, the TREC content of $CD4^+$ T_{TN} cells declines from 0.25 to 0.143, while that of $CD8^+$ T_{TN} cells only declines from 0.25 to 0.167. This figure was created using BioRender.

$CD95^+$ naive T cell population falls very high within the gate for conventional naive T cells (results not shown), suggesting that the conventional naive T cells of which the TREC content was determined, were already very pure to begin with. Nonetheless, by also filtering out the remaining $CD95^+$ naive T cells, we were able to determine the TREC content of the most pure form of human naive T cells. This allowed us to quantify the relative contribution of the thymus to the maintenance of the human *truly* naive T cell compartment. We found that ~22% of the $CD4^+$ and ~34% of the $CD8^+$ T_{TN} pool in healthy human adults had been produced by the thymus. Unsurprisingly, these numbers show that the human thymus contributes more to the production of $CD8^+$ than $CD4^+$ T_{TN} cells. It also confirms that the human *truly* naive T cell compartment is mainly maintained through peripheral division.

The $CD95^+$ naive T cell population ($CD45RO^-CD27^+CCR7^-CD95^+$), that we excluded to isolate human *truly* naive T cells, did most likely not consist purely of T_{SCM} cells, as T_{SCM} cells express both CCR7 and CD95. Remarkably, we did not find a clear T_{SCM} population in our human donors. It remains a question what type of T cell population the $CD95^+$ naive T cells do represent, but it is possible, also considering their relatively low TREC content in comparison to that of *truly* naive T cells, that these cells are transitioning from a naive to memory phenotype.

Where the human naive T cell compartment thus seems to be mostly maintained through peripheral division, it was previously established that naive T cells in SPF mice are almost exclusively produced through thymic output[8]. However, as it has been shown in mice that the level of antigen exposure may directly influence T cell dynamics, we wanted to re-evaluate this matter in a wildling mouse model. We started by looking for an effect of age on the thymus weight of wildlings. As it turned out, the thymus of wildlings decreases roughly three times between ten and twenty-nine weeks in age, much faster than the rate of thymic involution in normal SPF mice[23]. We also found that, similar to SPF mice, naive T cells in wildlings are mostly produced through thymic output. It thus appears that the maintenance of the naive T cell pool is differently regulated in humans and mice. Why the thymus plays a signficantly more prominant role in the production of naive T cells in mice than in humans remains to be elucidated. Wheras mice are born lymphopenic, humans already contain a competent T cell compartment at birth[24]. As humans encounter most new antigens early in life[1], it is important to have a broad range of different T cells during this period. The only way new T cell specificies are produced is via thymic output, not through peripheral division[8]. This could explain why the contribution of the thymus to human naive T cell production declines after adolescence is reached. One the other hand, mice might keep encountering a lot of different pathogens throughout their life, for example because they live in different surroundings than humans. This would mean that new clones of T cells need to keep being produced, explaining why the relative contribution of the thymus to naive T cell production remains high throughout the life of mice. Looking from a completely different angle, it might be possible that the threshold for inducing compensatory naive T cell divison lies much higher in mice than in humans. The thymus of humans as well as mice involutes throughout life, but where human naive T cell numbers remain relatively constant[6,7], naive T cell numbers in mice decrease[23]. It is thought that in humans, low naive T cell numbers as a result of thymic involution leads to increased peripheral division[5]. In mice, pheripheral naive T cells barely divide[8], which might be due to a lower sensitivity for stimuli inducing proliferation. Only when neonatal mice are fully thymetomized, naive T cells show some reduction in TREC content, suggesting peripheral division[8]. Whether murine naive T cells are indeed less sensitive to stimuli that are involved in the regulation of T cell proliferation, such as IL-7[25], remains to be investigated.

In this project, only wildlings from ten to twenty-nine weeks in age were included. It was unfortunately not feasible to include willdings that were older, however, these data will be collected in the future. Only then, a more definite conclusion can be drawn whether the way naive T cells are maintained is indeed species-specific. Although the immune phenotype of willdings closely resembles the immune phenotype of wild mice, they are still not the identical[17]. It can therefore not be fully excluded that differences exist in naive T cell dynamics between wildlings and wild mice. One might also argue that we did not include the most pure form of naive T cells from wildlings, as T_{SCM} cells also exist in mice[14]. However, assuming that, just like in humans[14], the murine T_{SCM} cell population contains a relatively low TREC content, exluding this population would only lead to a stronger increase in the TREC content of murine naive T cells with age. The conclusion that naive T cells in wildlings are mainly produced through thymic output would not be effected.

Indeed, the TREC content of naive T cells from wildlings signficantly increased with age. This is different from what has previously been observed in SPF mice, where the TREC content of naive T cells remained relatively stable and even declined slightly with age[8]. As the thymus of wildlings appears to involute even faster than the thymus of SPF mice, the increase in TREC content does not seem to be the result

of an increase in naive T cell production throughout the life of a willding mouse. We considered that the increase might be due to the existance of a separate population within the naive T cell compartment of wildlings. When this separate population would divide relatively slow compared to the rest of the naive T cell compartment, the TREC content of naive T cells would increase. However, as it has previously been shown that most naive T cells do not even divide during the life of a mouse[8], this seemed unlikely. It could be possible that a bias arises in the naive T cell population that migrates from the thymus to the spleen throughout the life of a wildling. If relatively more $TREC^+$ naive T cells enter the spleen in old wildlings than in young wildlings, this might explain the increase in the TREC content of naive T cells from the spleen.

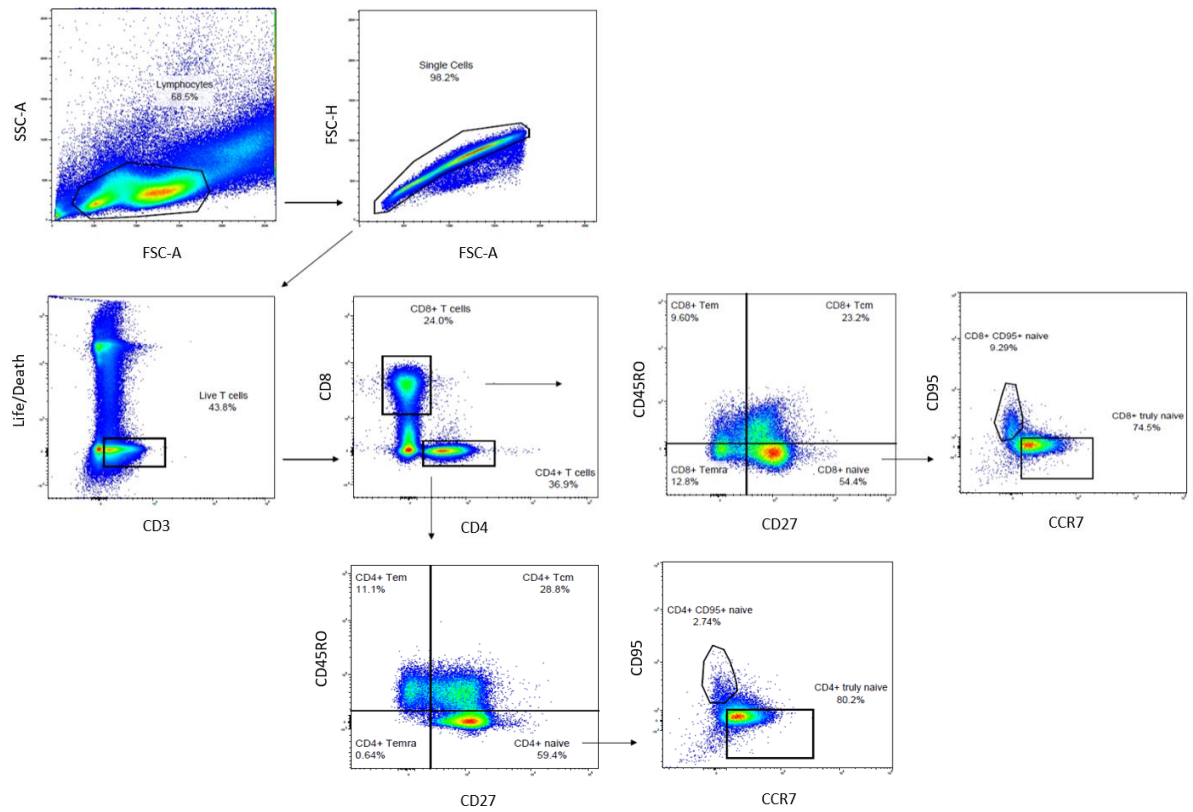
Collectively, we show that the maintenance of naive T cells is differently regulated in mice and men. Where the human *truly* naive T cell compartment is mainly maintained through peripheral division, naive T cells in wildlings are almost exclusively produced by the thymus. Although these findings tell us that using the mouse as a direct model for humans should be done with caution, further study is required to explore what could explain this difference in naive T cell maintenance.

Supplementary Methods

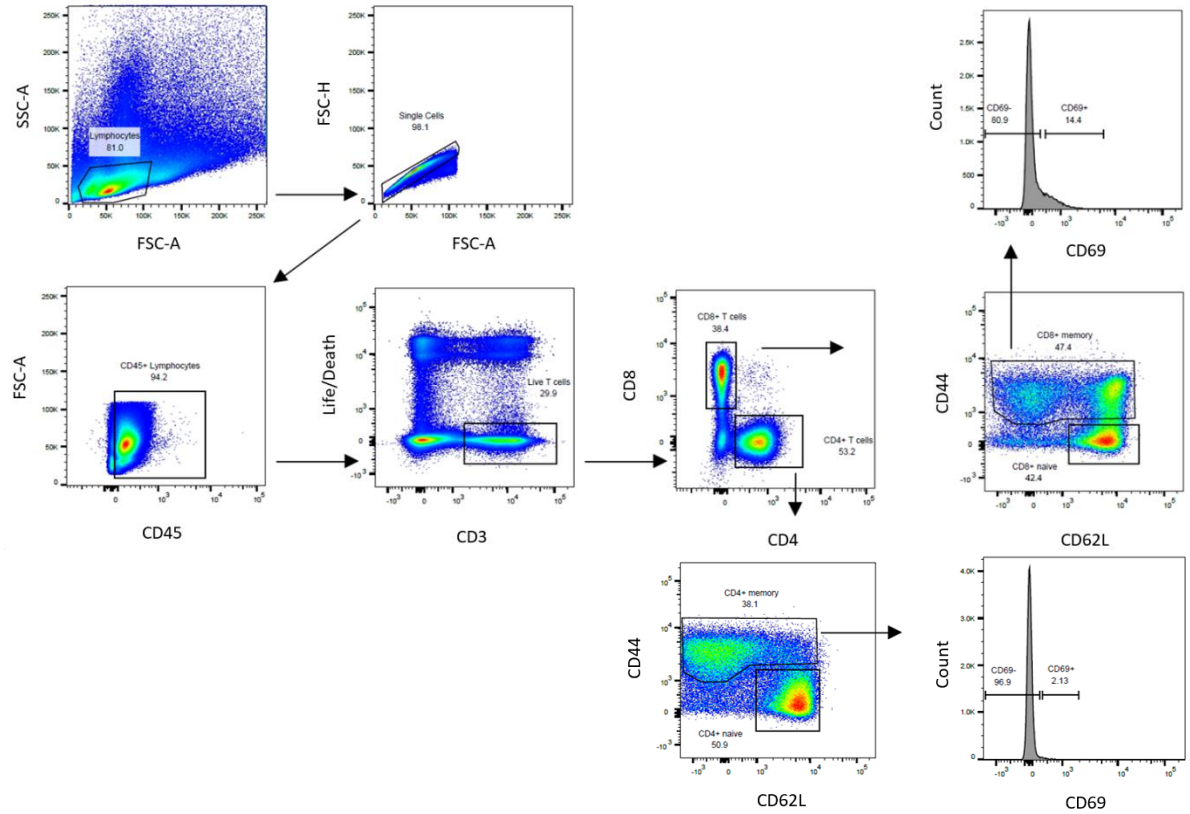
Bacterial transformation & sequencing sj TREC plasmid

For the sj TREC standard curve in the human TREC assays, we made use of a sj TREC plasmid. The sj TREC plasmid consisted of a pUC19 vector, cloned with the sj TREC fragment at the HindIII restriction site (Supplementary Figure 3)[6]. Bacterial transformation was conducted in order to obtain a sj TREC plasmid stock that could be used to create the sj TREC standard curve. In short: 5 µl sj TREC plasmid was added to 25 µl DH5α bacterium source, after which the bacteria were heat shocked at 42°C for 45sec. To achieve maximum transformation efficiency, 200 µl SOC medium (Thermo Fisher Scientific) was added to the bacterial suspension prior to an 1hour incubation step at 37°C. Following two overnight growth steps of the transformed bacteria at 37°C, the sj TREC plasmid was isolated using the PureLink™ HiPure Plasmid Filter Maxiprep Kit (Thermo Fisher Scientific). To verify whether the transformation was successful, the sj TREC plasmid stock was sequenced. The M13 forward primer 5'-GTAAAACGACGGCCAGT-3' and M13 reverse primer 5'-GGAAACAGCTATGACCATG-3' were used to sequence the sj TREC fragment. 5µL of the plasmid stock was added together with 5µL of either the M13 forward or reverse primer. The sequencing results are depicted in Supplementary Figure 3. The pUC19 vector itself has a length of 2686bp. From the sequencing results it can be derived that the sj TREC fragment has a length of 181bp, making the total length of the sj TREC plasmid 2867bp. The sj TREC forward and reverse primer are highlighted in blue and are exactly overlapping with the theoretical sequences of the primers described in the Material & Methods section. The same holds for the sj TREC probe, which is highlighted in red. We were provided with the following theoretical sequence of the sj TREC fragment: 5'-AAG CTT AGA GGG GTG CCT CTG TCA ACA AAG GTG ATG CCA CAT CCC TTT CAA CCA TGC TGA CAC CTC TGG TTT TTG TAA AGG TGC CCA CTC CTG TGC ACG GTG ATG CAT AGG CAC CTG CAC CCC GTG CCT AAA CCC TGC AGC TGG CAC GGG CCC TGT CTG CTC TTC ATT CAC CGT TCT CAC GAG TTG CAA AAG CTT-3'. The bases AAGCTT represent the HindIII restriction site of the pUC19 plasmid and should thus be located at the beginning and end of the sj TREC gene insert. Comparing the above mentioned theoretical sequence of the sj TREC fragment with the sequencing results, we can observe that the sequences are mostly overlapping, except for the last couple of bases. The HindIII restriction site that, according to the theoretical sequence, should be located at the end of the sj TREC gene fragment, is missing. This explains why we were not able to cut the sj TREC gene insert out of the pUC19 plasmid using HindIII restriction enzymes and visualize it on an agarose gel (results not shown). The sequencing results also show that from the start of the sj TREC forward primer until the end of the sj TREC reverse primer, the sequence is exactly similar to the theoretical sequence of the sj TREC gene insert. As this is the sequence that is multiplied in the human TREC assays, it is crucial that this part of the sequence is correct. It is unknown why the sequence of the sj TREC fragment in the sj TREC plasmid we are using is slightly different from the theoretical sequence of the sj TREC fragment.

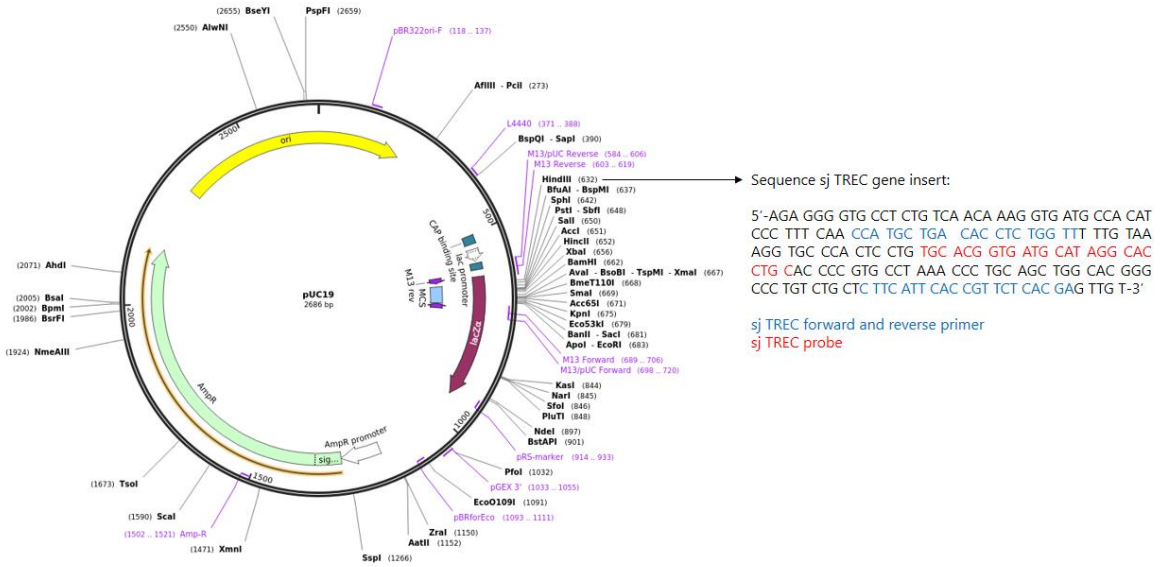
Supplementary Figures



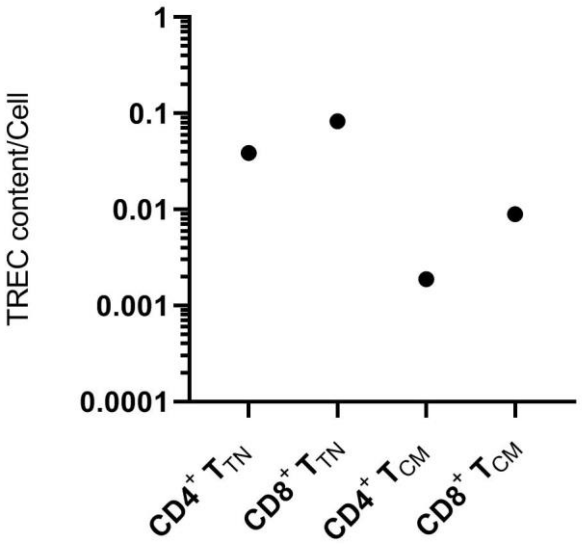
Supplementary Figure 1: Representative gating strategy for human $CD4^+$ and $CD8^+$ T_{TN} cells, $CD95^+$ naive T cells and T_{CM} cells. PBMCs from healthy individuals were stained with CD3-AF700, CD8a-PerCP-Cy5.5, CD4-BV785, CD45RO-BV711, CCR7-PE-Cy7 (Biolegend), CD27-BV510 and CD95-APC (BD Biosciences), and sorted to obtain T_{TN} cells ($CD45RO^-CD27^+CCR7^+CD95^-$), $CD95^+$ naive T cells ($CD45RO^-CD27^+CCR7^-CD95^+$) and T_{CM} cells ($CD45RO^+CD27^+$).



Supplementary Figure 2: Representative gating strategy for murine $CD4^+$ and $CD8^+$ naive and memory T cells. Fresh splenocytes from wildlings were treated with Fc-Block (BD Biosciences) and stained with CD45-APC-eF780 (eBioscience), CD3e-PE-Cy5, CD44-BV605 (Biolegend), CD8a-BV786, CD4-AF700, CD62L-BV650 and CD69-Pe-Cy7 (BD Biosciences), and sorted to obtain naive ($CD62L^+CD44^-$) and $CD69^-$ memory T cells ($CD44^+CD69^-$).



Supplementary Figure 3: The sj TREC plasmid, consisting of a pUC19 vector cloned with the sj TREC gene fragment at the HindIII restriction site. The pUC19 vector itself has a length of 2686bp. The sj TREC gene insert has a length of 181bp, making the total length of the sj TREC plasmid 2867bp. The sj TREC forward and reverse primer are highlighted in blue, the sj TREC probe is highlighted in red. We confirmed the sj TREC gene insert by sequencing the plasmid using the M13 forward and reverse primer.



Supplementary Figure 4: The TREC content of T_{CM} cells is lower compared to the TREC content of T_{TN} cells. The TREC content per cell is depicted for paired $CD4^+$ and $CD8^+$ T_{TN} and T_{CM} cells.

Donor	Age (years)	Proliferation rate **CD4⁺** T_{TN} cells	**CD4⁺** T_{TN} cell count/mL blood ($x10^6$)	Total amount of **CD4⁺** T_{TN} cells in the body ($x10^{10}$)	Proliferation rate **CD8⁺** T_{TN} cells	**CD8⁺** T_{TN} cell count/mL blood ($x10^6$)	Total amount of **CD8⁺** T_{TN} cells in the body ($x10^{10}$)
DICE E04	70	0.000174265	0.212	5.31	0.000121114	0.157	3.92
DICE E08	67	0.001813506	0.137	3.44	0.0021563	0.037	0.93
DICE E10	71	0.001370971	0.389	9.73	0.001371693	0.230	5.74
DICE E21	70	0.000683642	0.196	4.90	0.000718711	0.066	1.64
DICE E23	71	0.000626299	0.116	2.91	0.000139554	0.081	2.03
DICE E30	67	0.001666685	0.828	20.69	0.000966302	0.397	9.92
DICE E34	68	0.000431429	0.192	4.80	0.000492153	0.045	1.12
DICE E35	70	0.001793215	0.432	10.8	0.001278411	0.216	5.40
DICE E39	67	0.000302028	0.086	2.16	3.11819E-05	0.021	0.53
DICE E59	76	0.000105186	0.459	11.50	0.000611861	0.031	0.76
Median		0.000654971	0.204	5.11	0.000665286	0.073	1.84

Supplementary Table 1: Proliferation rate p, cell count/mL blood and total amount of cells in the body for human CD4⁺ and CD8⁺ T_{TN} cells. The proliferation rate p of T_{TN} cells was estimated through ^2H_2O labeling (Van den Berg *et al.* (submitted)). The total amount of CD4⁺ and CD8⁺ T_{TN} cells in the body was calculated as follows: (amount of CD4⁺/CD8⁺ T_{TN} cells/mL blood (Van den Berg *et al.* (submitted)) x 5000 (5L whole blood) x 50 (assuming that only 2% of the lymphocytes reside in the blood[8]). These values were used to calculate the daily thymic output of human CD4⁺ and CD8⁺ T_{TN} cells (Table 1).

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