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Article

Maintenance of Peripheral Naive T Cells Is Sustained by Thymus Output in Mice but Not Humans

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SUMMARY

Parallels between T cell kinetics in mice and men have fueled the idea that a young mouse is a good model system for a young human, and an old mouse, for an elderly human. By combining in vivo kinetic labeling using deuterated water, thymectomy experiments, analysis of T cell receptor excision circles and CD31 expression, and mathematical modeling, we have quantified the contribution of thymus output and peripheral naive T cell division to the maintenance of T cells in mice and men. Aging affected naive T cell maintenance fundamentally differently in mice and men. Whereas the naive T cell pool in mice was almost exclusively sustained by thymus output throughout their lifetime, the maintenance of the adult human naive T cell pool occurred almost exclusively through peripheral T cell division. These findings put constraints on the extrapolation of insights into T cell dynamics from mouse to man and vice versa.

INTRODUCTION

Insights into human T cell dynamics, whether in healthy aging or during lymphopenia, have been largely based on experiments in mice. For example, slow naive T cell recovery in lymphopenic humans is thought to be caused by age related thymic atrophy (Crooks et al., 2006), and long-term failure of immune reconstitution after stem cell transplantation (SCT) has been related to exhaustion of thymic output (Patel et al., 2000; Sarzotti et al., 2003). Both interpretations were influenced by the important role of thymic output in naive T cell generation in mice. Likewise, thymic failure has been suggested to be important in $CD4^+$ T cell loss during HIV infection (Douek et al., 2001), and rapid thymic rebound is proposed to be responsible for T cell reconstitution in HIV infected patients during antiviral treatment (Dion et al., 2004). The reason why insights into T cell dynamics are so widely extrapolated from mice to men and vice versa is that there are clear parallels between these species. In both species, thymus output declines with age (Hale et al., 2006; Steinmann et al., 1985), which is probably responsible for the gradual decline in naive $CD4^+$ and $CD8^+$ T cell numbers, although naive T cell numbers decline less dramatically than thymocyte numbers (Fagnoni et al., 2000; Sempowski et al., 2002). At old age the peripheral T cell pool becomes dominated by memory T cells (Fagnoni et al., 2000; Lerner et al., 1989), coinciding with severe perturbations of the naive T cell repertoire and impaired immunity (Ahmed et al., 2009; Naylor et al., 2005; Yager et al., 2008). Together, such parallels have fueled the idea that a young adult mouse is a good model system for a young adult human, and an old mouse, for an elderly human. In fact, there is no formal proof that such extrapolations are justified (Mackall and Gress, 1997).

Using a unique combination of state of the art techniques in mice and men, we have directly quantified the contribution of de novo T cell production by the thymus and peripheral T cell division in mice and men. On the basis of T cell receptor excision circle (TREC) data, the byproducts of V(D)J recombination in the thymus (Douek et al., 1998), we estimated that in healthy human adults ~90% of the naive $CD4^+$ T cell pool has been generated by peripheral T cell proliferation. By labeling mice with deuterated water (^2H_2O) (Neese et al., 2002), we estimated that the average life span of murine naive $CD4^+$ and $CD8^+$ T cells throughout life is ~7 and 11 weeks, respectively, which is ~40-fold shorter than in humans (Vrisekoop et al., 2008). Analyses of the change in naive T cell numbers after thymectomy revealed that in mice, naive T cells are almost exclusively derived from thymus output, even at very old age.

In summary, our data show that mice and men are incomparable with respect to naive T cell maintenance because the major source by which naive T cell numbers are maintained is fundamentally different in mice and men. These results have obvious limitations for mouse experiments that aim to understand, e.g., T cell reconstitution in lymphopenic patients or the effects of aging in healthy humans.

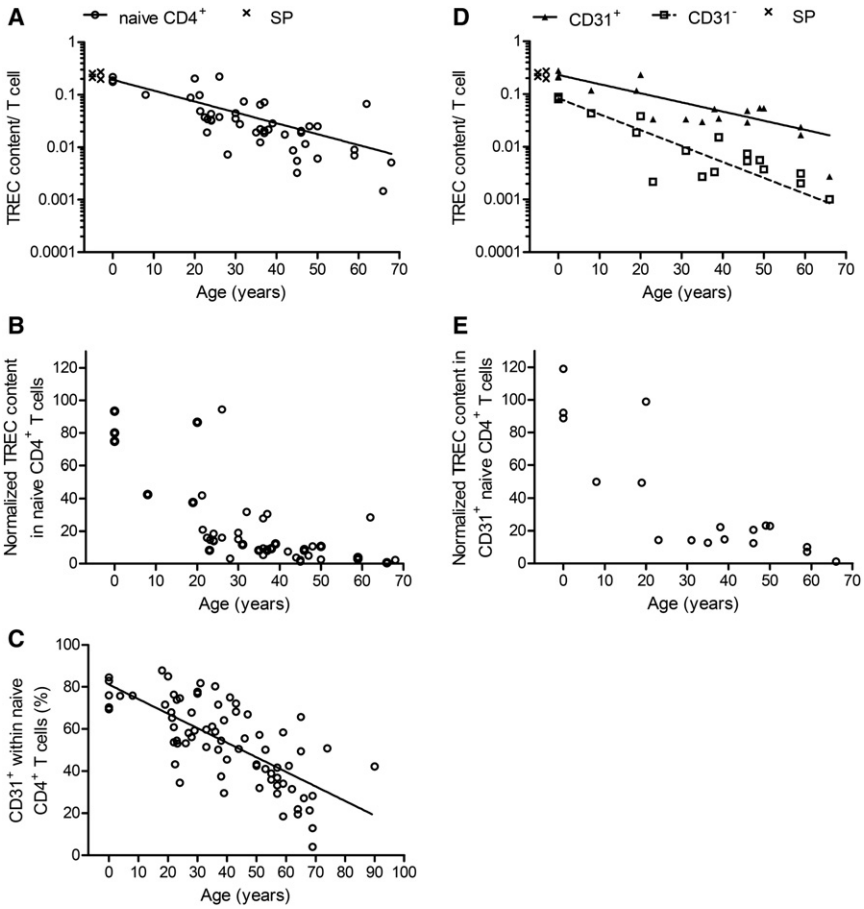

Figure 1. Quantification of the Contribution of Thymic Output in Humans

(A) TREC content of naive $CD4^+$ T cells as a function of age (n = 45).

(B) Percentage of naive $CD4^+$ T cells that were originally produced in the thymus, calculated by normalizing the TREC content of peripheral naive $CD4^+$ T cells by the TREC content of SP $CD4^+$ thymocytes (n = 45).

(C) The percentage of $CD31^+$ T cells within the naive $CD4^+$ T cell pool of healthy individuals (n = 76) decreased significantly ($R_p = -0.72$, $p < 0.001$) with age.

(D) TREC contents of $CD31^+$ (▼) and $CD31^-$ (□) naive $CD4^+$ T cells in healthy donors (n = 18) of different ages. The TREC content of both $CD31^+$ naive $CD4^+$ T cells ($R_s = -0.78$, $p < 0.001$) and $CD31^-$ naive $CD4^+$ T cells ($R_s = -0.80$, $p < 0.001$) declined significantly with age; their slopes were not significantly different (p = 0.25). TREC contents of $CD31^+$ cord blood $CD4^+$ T cells (n = 3) were similar to TREC contents of single-positive $CD4^+CD8^-$ thymocytes (n = 4, p = 0.86).

(E) The percentage of $CD31^+$ naive $CD4^+$ T cells that were originally produced in the thymus, calculated by normalizing the TREC content of $CD31^+$ naive $CD4^+$ T cells in the blood by the TREC content of SP $CD4^+$ thymocytes (n = 18).

RESULTS

Contribution of Thymic Output to the Human Naive T Cell Pool

To quantify the relative contribution of thymic output in humans, we measured the TREC content of naive $CD4^+$ T cells in healthy individuals of different ages (Figure 1A). We have previously argued that TREC contents cannot be used as a measure for daily thymic output because the TREC content of a T cell population increases with thymic output and with cell loss and declines with cell division (De Boer, 2006; Dutilh and de Boer, 2003; Hazenberg et al., 2000). Given that TRECs are not copied during peripheral proliferation, each TREC remains a true marker of thymic origin, and the number of TREC-positive cells in a population reflects the number of cells that were produced by the thymus at any point in time and that are still present in the periphery. Conversely, the number of TREC-negative naive T cells reflects the number of cells in the population that have been produced by peripheral proliferation. The average TREC content of a naive T cell population can therefore be used to estimate the fraction of cells that were originally produced by the thymus.

One complication is that only a fraction of the cells leaving the thymus is actually carrying a TREC. To estimate the fraction of cells that originated from the thymus, one therefore has to normalize the observed TREC content by the average TREC content of a recent thymic emigrant (RTE). Thanks to the fact that the average TREC content of thymocytes does not decrease with age (Jamieson et al., 1999), we could estimate the TREC content of RTEs by measuring TRECs in single positive (SP) thymocytes from children who underwent cardiac surgery. Along with these samples, we measured TRECs in naive $CD4^+$ T cells from healthy volunteers of different ages. When these peripheral TREC contents were normalized to the average TREC contents of $CD4^+$ SP thymocytes, we found that the median fraction of naive $CD4^+$ T cells that were originally produced by the thymus in adults was 11% (see Figure 1B). Thus, ~90% of the naive T cell pool in these adults had been formed by peripheral naive T cell proliferation.

Contribution of Peripheral Proliferation to the Human $CD31^+$ Naive $CD4^+$ T Cell Pool

Naive $CD4^+$ T cells expressing CD31 (PECAM-1) are thought to be enriched in cells that were produced by the thymus (Kimmig et al., 2002; Kohler et al., 2005). In agreement with previous studies (Kilpatrick et al., 2008; Kimmig et al., 2002; Kohler et al., 2005), we found that the fraction of $CD31^+$ T cells within the naive $CD4^+$ T cell pool of healthy individuals decreased substantially—and almost linearly—with age (Figure 1C) and that the $CD31^+$ naive $CD4^+$ T cell population always had a higher TREC content than the $CD31^-$ population. The average TREC contents of $CD31^+$ and $CD31^-$ naive $CD4^+$ T cells declined substantially and at similar rates with age (Figure 1D), confirming that even $CD31^+$ naive $CD4^+$ T cells are in part produced by peripheral T cell division (Kilpatrick et al., 2008).

Table 1. Total and Thymic Daily Naive T Cell Production in Young Human Adults

Individual	Age (years)	Normalized Naive CD4+ TREC Content[a]	Total Daily Naive CD4+ T Cell Production (× 10^7) (cells/day)[b]	Daily CD4+ Thymic Output (× 10^7) (cells/day)[c]
A	24	14%	12	1.6
B	22	16%	5.4	0.85
C	25	ND	2.2	ND
D	20	21%	8.2	1.7
E	22	15%	13	1.9

[a] Normalized naive CD4+ TREC content, calculated by dividing the TREC content of peripheral naive CD4+ T cells by the TREC content of SP CD4+ thymocytes.
[b] Total production of naive CD4+ T cells per day, calculated as p × (the naive T cell count per liter blood) × (5 l blood) × 50, assuming that 2% of lymphocytes reside in the blood (Westermann and Pabst, 1990), where p is the average turnover rate estimated by ^2H_2O labeling (Vrisekoop et al., 2008).
[c] Daily CD4+ thymic output, calculated by multiplying the normalized naive CD4+ TREC content with the total daily naive CD4+ T cell production.

To quantify which fraction of CD31+ naive CD4+ T cells originated from peripheral renewal, and which from thymic output, we normalized the TREC content of CD31+ naive CD4+ T cells to the average TREC content of SP CD4+CD8− thymocytes. The TREC content of CD31+ naive CD4+ T cells in cord blood turned out to be very similar to the TREC content of SP CD4+ CD8− thymocytes, suggesting that cord blood CD31+ naive CD4+ T cells had not markedly proliferated since they emerged from the thymus, and that the average TREC content of SP thymocytes was indeed representative for the TREC content of RTE. We found that the percentage of naive CD31+ CD4+ T cells that had originally been produced in the thymus decreased with age, from a median of >99% in neonates to 23% in adults (see Figure 1E). Thus, even the vast majority of CD31+ naive CD4+ T cells in human adults are formed by peripheral proliferation.

Daily Thymic Output in Humans

From the basic model for TREC and naive T cell dynamics (Hazenberg et al., 2000) one can deduce that, in equilibrium, the normalized TREC content (A/c) of naive T cells is given by:

$$\frac{A}{c} = \frac{\sigma(t)}{\sigma(t) + pN(t)} \quad (1)$$

where A is the TREC content of naive T cells, c is the TREC content of SP thymocytes, $\sigma(t)$ represents thymic output at time t, p is the rate of naive T cell proliferation, and $N(t)$ is the number of naive T cells at time t. This equation confirms that the TREC content of two individuals with the same thymic output may be totally different if they differ in their peripheral renewal $pN(t)$ (Hazenberg et al., 2000). If the current total daily production of naive T cells $\sigma(t) + pN(t)$ is known, Equation 1 can be used to obtain an upper estimate of the number of cells $\sigma(t)$ exported by the thymus. In five healthy volunteers we previously quantified the total daily production of naive T cells using deuterium labeling (Vrisekoop et al., 2008, see Table 1). Combining these estimates with the normalized TREC contents of naive CD4+ T cells in these individuals revealed a median daily thymic output of 1.7 × 10^7 CD4+ T cells per day (see Table 1).

Turnover of Naive T Cells in Mice

To compare the above insights obtained in humans to T cell dynamics in mice, we determined the normal turnover rates of naive CD4+ and CD8+ T cells in young adult mice. To this end, we administered a bolus of 99.8% deuterated water (^2H_2O) to 12-week-old C57Bl/6 mice and then performed long-term maintenance labeling with 4% deuterated water in the drinking water. Deuterium enrichment in the DNA of thymocytes (Figure S1) and naive T cells from the spleen (Figure 2) was determined during 4 weeks of label administration and a subsequent 18 weeks of downlabeling period. It took approximately a week for the first labeled naive T cells to appear in the spleen, where they kept on accumulating up to a week after the end of labeling (Figure 2A), suggesting that these cells were labeled by cell division in another compartment.

The cellular turnover rates were estimated by fitting the labeling data to a mathematical model (Asquith et al., 2002; Vrisekoop et al., 2008), which distinguishes between an average turnover or production rate, p, and a death rate of labeled cells, d^* (see Experimental Procedures and Table S1). The average turnover rates of naive CD4+ and CD8+ T cells in young adult mice were found to be 0.021 and 0.013 per day (Figure 2A), corresponding to average life spans ($1/p$) of 47 and 80 days, respectively. In contrast to what is commonly observed in deuterium-labeling studies in man (Asquith et al., 2002), the death rates of labeled cells, d^*, did not significantly differ from the average turnover rates, p (Figure 2A), suggesting that naive T cells form a dynamically homogeneous population in the mouse.

Thymus Output in Mice

Although deuterium-labeling experiments provide the most reliable tool for estimating cellular turnover rates, they fail to distinguish between production of naive T cells in the thymus and their peripheral renewal (Borghans and de Boer, 2007). In a separate set of experiments, we therefore enumerated SP CD4+ and CD8+ thymocytes and naive CD4+ and CD8+ T cells in the spleen and PLNs of normal euthymic or sham thymectomized mice and mice that had been thymectomized at 7 weeks of age (Figure 3). There were no significant differences in thymocyte or naive T cell numbers between normal mice and sham thymectomized mice at any age (data not shown), allowing us to combine the data from both types of mice in one euthymic control group. In euthymic mice, the numbers of SP CD4+ and CD8+ thymocytes were found to increase exponentially after birth, after which they peaked at week 6–7 and decreased by

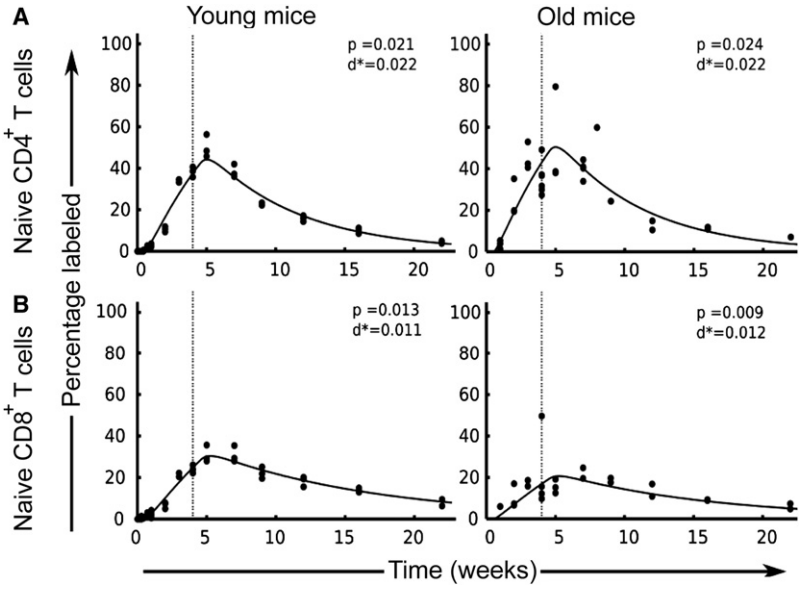

Figure 2. Estimating Mouse Naive T Cell Turnover with Deuterium Labeling

Twelve-week-old (n = 28, A) and 85-week-old mice (n = 28, B) were given 4% ^2H_2O for 4 weeks. Each dot represents the normalized deuterium enrichment in the DNA of naive $CD4^+$ (upper graphs) or naive $CD8^+$ T cells (lower graphs) in the spleen of one C57Bl/6 mouse. Vertical lines mark the end of ^2H_2O administration at 4 weeks. The estimated average turnover rates (p) and death rates of the labeled cells (d^*) resulting from the best fits of the mathematical model to the data are given in each graph.

almost 40% during week 7 (data not shown). Thereafter, thymocyte numbers declined exponentially at a rate of 50% per year (see Experimental Procedures). Naive T cell numbers in spleen and PLNs peaked at week 7–8 (see Figure 3) and subsequently declined more slowly than thymocyte numbers, suggesting that a homeostatic mechanism compensated for loss of thymus output.

Homeostatic Compensation through Decreased Peripheral Cell Death

A likely homeostatic mechanism by which cell numbers are regulated is a cellular survival rate that depends on the population density (Freitas and Rocha, 2000). When T cell numbers are low, T lymphocytes will experience less competition for survival signals, such as contact with MHC-peptide ligands or cytokines, and may thus have a longer life expectancy. To study the relative contribution of thymus output and peripheral T cell proliferation to the maintenance of the naive T cell pool in mice, we fitted a mathematical model to the naive T cell counts of euthymic and thymectomized mice of different ages that were measured experimentally. The model describes thymus output, peripheral T cell renewal, and naive T cell loss, which represents both cell death and priming of naive T cells into the memory T cell pool and depends on the number of T cells present (see Experimental Procedures and Table S2). The naive $CD4^+$ and $CD8^+$ T cell counts of euthymic and thymectomized mice turned out to be described very well with a simple model that totally lacks peripheral renewal of naive T cells and only allows for an increase in the average life span when T cell numbers decline (Figure 3; Table 2).

According to this "homeostatic survival" model, the average life spans of naive $CD4^+$ and $CD8^+$ T cells in 12-week-old mice are 31 and 72 days, respectively, which is in reasonable agreement with the 47 and 80 day expected life spans estimated

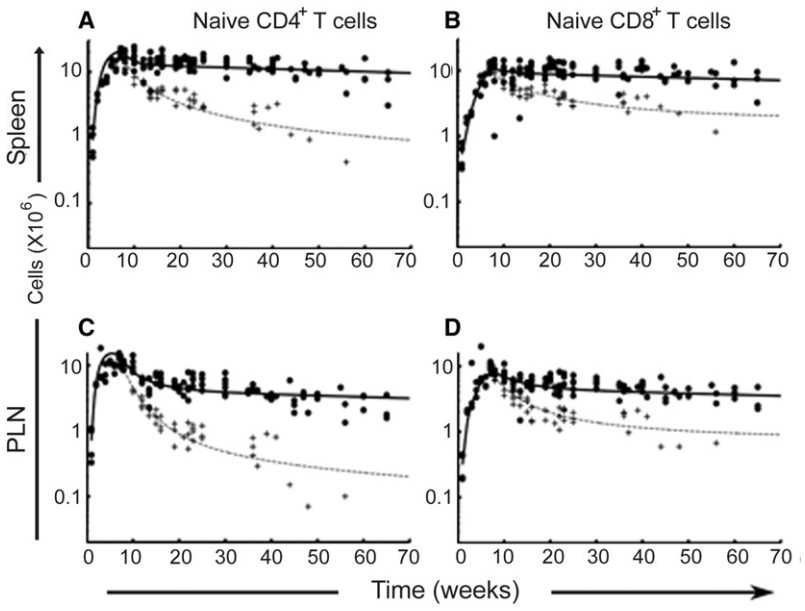

Figure 3. Effect of Thymectomy on the Size of the Naive T Cell Population in Mice

Numbers ($\times 10^6$) of naive $CD4^+$ (A and C) and naive $CD8^+$ T cells (B and D) were determined in spleen (A and B) and PLNs (C and D) of euthymic mice (●, n = 130) and mice that had been thymectomized at week 7 (+, n = 43). Population densities in control and thymectomized mice were fitted with a mathematical model in which the cellular death rate increased linearly with the population density (model 1; see Experimental Procedures). The best fits of the model to the combined data sets of spleen and lymph nodes of normal and thymectomized mice are depicted by the continuous and dotted curves, respectively. Best fitting parameters are given in Table 2 and corresponding average life spans are given in Table 3.

Table 2. Parameters Values of the Two Mathematical Models Describing Naive T Cell Numbers.

	Parameter	Value (confidence limits)[a]
Model 1: Homeostatic Survival[b]		
CD4$^+$	d_n ($\times 10^{-9}$ day^{-1})	2.5 (2.3–2.7)
	r_n (day^{-1})	0
CD8$^+$	d_n ($\times 10^{-9}$ day^{-1})	1.5 (1.4–1.6)
	r_n (day^{-1})	0
Shared[c]	ε (day^{-1})	0.034 (0.032–0.036)
Model 2: Homeostatic Proliferation		
CD4$^+$	d_n (day^{-1})[d]	0.021
	r_n (day^{-1})[e]	1
	h ($\times 10^4$ cells)	2.4 (1.8–2.4)
CD8$^+$	d_n (day^{-1})[d]	0.013
	r_n (day^{-1})[e]	1
	h ($\times 10^4$ cells)	3.0 (2.6–3.5)
Shared[c]	ε (day^{-1})	0.022 (0.021–0.023)

[a] 95% confidence intervals.
[b] Values are estimates of the best fit depicted in Figure 3.
[c] These parameters were forced to be equal when fitting CD4$^+$ and CD8$^+$ data.
[d] Parameter fixed to deuterium enrichment estimate.
[e] The best fit of the model gives a maximal renewal rate $r_n = 1$ for both CD4$^+$ and CD8$^+$ naive T cells.

Table 3. Estimated Average Life Spans of Naive CD4$^+$ and CD8$^+$ T Cells in Euthymic and Thymectomized Mice

Method	Age	Average Life Spans (days)[a]	
		Naive CD4$^+$	Naive CD8$^+$
^2H_2O	12 weeks	47 (41–54)[b]	80 (67–92)
	85 weeks	41 (36–47)	116 (94–139)
Cell counts	7 weeks	23 (22–25)	70 (63–78)
	12 weeks	31 (29–33)	72 (65–75)
	85 weeks	46 (43–49)	101 (94–105)
	12 weeks (ATx)	62 (61–64)	113 (105–117)

[a] The average life spans of young adult and old mice were based on the estimated turnover rate (life span = 1/p) from the deuterium-labeling experiments (^2H_2O, see Figure 2) and on the combination of estimated parameters from the homeostatic survival model (see Figure 3) and the T cell counts at the indicated ages (cell counts; where the average life span is defined as $1/(r+d_nN)$ and where N is the average number of naive T cells at the indicated age).
[b] 95% confidence limits are given in parentheses.

from the deuterium-labeling experiments (Table 3). Because the export of naive T cells from the thymus is proportional to the number of SP thymocytes (Berzins et al., 1998), we estimated that every day 3.4% (3.2%–3.6%) of the SP thymocyte pool emigrates from the thymus to the spleen (Table 2). In 12-week-old mice, this corresponds to a daily emigration of 3.6×10^5 (confidence interval: 1.3×10^5–6.2×10^5) newly produced naive CD4$^+$ and 1.5×10^5 (confidence interval: 0.7×10^5–2.3×10^5) naive CD8$^+$ T cells from the thymus to the spleen. These estimates were solely derived from naive T cell numbers in euthymic and thymectomized mice. From the ^2H_2O-labeling experiments in euthymic mice described above, we independently calculated the total daily production of naive T cells by multiplying the average turnover rates (p) with the actual number of splenic naive T cells (N), yielding a total daily production of 2.7×10^5 (confidence interval: 0.9×10^5–4.9×10^5) naive CD4$^+$ and 1.2×10^5 (confidence interval: 0.2×10^5–2.2×10^5) naive CD8$^+$ T cells in the spleen (Figure S2A). Because total daily production and daily thymic output are so similar, we conclude that naive CD4$^+$ and CD8$^+$ T cells in young adult C57Bl/6 mice are almost entirely thymus derived and are hardly formed by peripheral T cell proliferation.

Homeostatic Compensation through Increased Peripheral Cell Division

Because some studies have suggested that T cell proliferation rates may increase when T cell numbers decrease (Surh and Sprent, 2008), we also analyzed the data with an alternative model, in which the rate of T cell proliferation increases when cell numbers are low. The fit of this alternative model to the data of euthymic and thymectomized mice was almost as good as the fit with the model in which T cell death rates are dependent on T cell densities. T cell proliferation rates resulting from these analyses were used for calculating average interdivision times. In normal 12-week-old mice, the average time between T cell divisions was estimated to be 218 and 655 days for naive CD8$^+$ and CD4$^+$ T cells, respectively. Comparison of the estimated average life spans from the deuterium-labeling experiments with these average interdivision times shows that the average time it takes for naive CD8$^+$ and CD4$^+$ T cells in euthymic mice to divide is 3- to 14-fold longer than the expected lifespan of these cells, implying that during their stay in the naive T cell pool most naive T cells never divide.

T Cell Dynamics in Old Mice

Knowing that naive T cell production in young adult mice is almost exclusively due to T cell production by the thymus, we studied naive T cell life spans in old mice which have much lower thymus output. To this end, we performed deuterium-labeling experiments in 85-week-old mice (Figure 2B). Naive CD4$^+$ T cells in aged mice were found to have an average life span of 41 days, which did not significantly differ from that in 12-week-old mice (47 days). The expected life span of naive CD8$^+$ T cells was found to be 116 days, which is nearly 50% longer than that in young adult mice (80 days, Table 3). Total daily splenic naive CD4$^+$ T cell production ($p \times N$) of old mice was on average 1.4×10^5 (confidence interval: 0.4×10^5–2.9×10^5) cells, of which 0.9×10^5 (confidence interval: 0.4×10^5–1.4×10^5) were produced by the thymus (Figure S2B). Similarly, 0.3×10^5 (confidence interval: 0.1×10^5–0.5×10^5) of the 0.6×10^5 (confidence interval: 0.1×10^5–1.5×10^5) naive CD8$^+$ T cells produced per day in old mice were produced by the thymus (Figure S2B). Thus, even in old mice, in which thymus output has dropped significantly, naive CD4$^+$ and CD8$^+$ T cell proliferation hardly contributes to naive T cell maintenance.

TREC Dynamics in Aging Mice

The result that peripheral T cell proliferation hardly contributes to the maintenance of the naive T cell pool throughout the life of

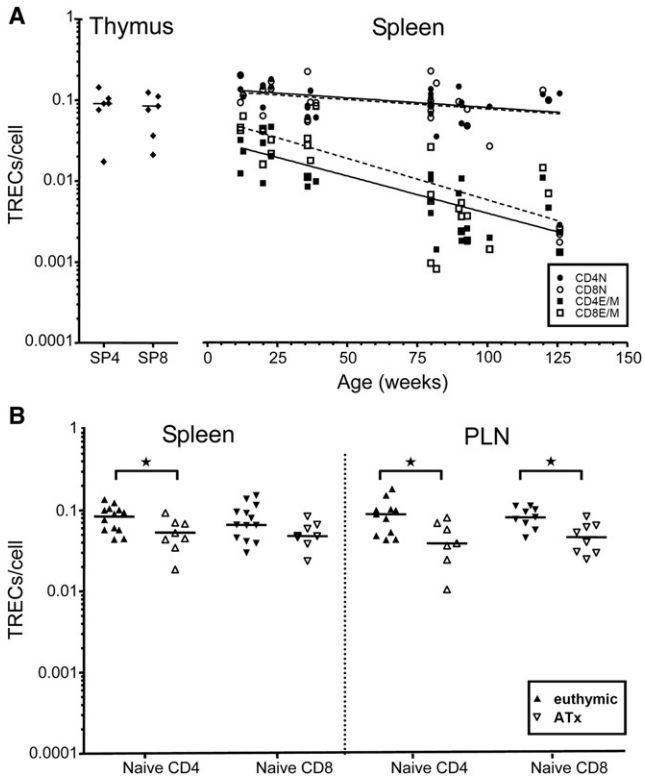

Figure 4. TREC Analysis in Mouse Thymocytes and Peripheral T Cell Subsets

(A) The average number of TRECs per T cell (on a logarithmic scale) from the spleens of mice ranging from 12 to 126 weeks of age (n = 29). Lines show the regression analyses through the data in each T cell subset. TREC contents of SP $CD4^+$ ($p = 0.13$) and SP $CD8^+$ ($p = 0.60$) thymocytes did not change with age (not shown) and are plotted separately. TREC contents of naive $CD4^+$ (●, $p = 0.11$) and naive $CD8^+$ (○, $p = 0.46$) T cells did not decrease significantly with age, whereas TREC contents of memory $CD4^+$ (■, $p = 0.0002$, $R^2 = 0.56$) and memory $CD8^+$ (□, $p = 0.0001$, $R^2 = 0.56$) T cells did. Analyses in LNs from the same mice revealed similar data (not shown).

(B) TREC contents of naive $CD4^+$ and $CD8^+$ T cells in age matched healthy control (▼, n = 13) and thymectomized mice (ATx at week 7, ∇, n = 8). Horizontal bars depict median values. TREC contents of naive $CD4^+$ T cells in the spleen ($p = 0.023$) and naive $CD4^+$ ($p = 0.024$) and naive $CD8^+$ ($p = 0.011$) T cells in the LNs were significantly lower in ATx mice compared to healthy controls.

a mouse is in sharp contrast with observations on naive TREC dynamics in humans (see Figures 1A and 1B; Harris et al., 2005; Kilpatrick et al., 2008; Prelog et al., 2009; Ribeiro and de Boer, 2008). An experimental prediction that naturally follows from our results is that—in contrast to what is observed in humans—the fraction of TREC-positive naive $CD4^+$ and $CD8^+$ T cells in healthy mice should not decrease with age. We tested this prediction by comparing the average TREC contents of naive and memory $CD4^+$ and $CD8^+$ T cells from normal euthymic mice between 12 and 126 weeks of age. We found no evidence for TREC dilution in mouse naive T cells with age, even though the average TREC contents of memory $CD4^+$ and $CD8^+$ T cells clearly declined with age (Figure 4A). The average TREC contents of naive $CD4^+$ and $CD8^+$ T cells throughout life were very similar to the average TREC contents of $CD4^+$ and $CD8^+$ SP thymocytes, confirming that naive T cells in mice hardly divide. Taken together, this is independent experimental confirmation of our main finding that—irrespective of their age—naive $CD4^+$ and $CD8^+$ T cells in euthymic mice are almost exclusively formed by thymus output. Interestingly, in thymectomized mice the fractions of TREC-positive naive T cells were moderately decreased (Figure 4B), suggesting that in the absence of the thymus, naive T cells in mice may ultimately proliferate.

DISCUSSION

We here show that the source by which naive T cell numbers are maintained during aging differs fundamentally between mouse and man. Not only in young adult but even in very old mice, the vast majority of the naive T cell pool is sustained by thymic output, whereas in human adults, the majority of naive cells are produced by peripheral T cell proliferation. Our results are in line with studies in lymphopenic humans and mice receiving bone marrow transplantation, which collectively suggested that T cell reconstitution in adult humans is more comparable to that in thymectomyzed mice than that in euthymic mice (Mackall and Gress, 1997). It is important to realize that these quantitative differences between mouse and man will also have qualitative effects on the naive T cell pool because the thymus is capable of producing new T cell specificities, whereas peripheral T cell proliferation can only lead to the expansion of already existing T cell clones.

The contribution of the thymus to the maintenance of the naive T cell pool in healthy adults and its potential to reconstitute the T cell pool in lymphopenic individuals has been much debated. Although some have ascribed a crucial role to the thymus in human adults (Douek et al., 1998), others have argued that during adulthood, the thymus is producing too few T cells to have a significant effect on the size of the naive T cell pool (Hazenberg et al., 2000). Our data point out that the vast majority of naive T cells in human adults are maintained through peripheral T cell proliferation. Several recent studies have suggested that even in young, healthy children, a substantial proportion of naive T cells are derived from peripheral T cell renewal (Bains et al., 2009; Hazenberg et al., 2004).

Our current estimates of the contribution of thymic output to the total daily production of naive $CD4^+$ T cells were much lower than previous estimates based on CD31 expression alone (Kilpatrick et al., 2008). Although the fraction of $CD31^+$ T cells within the naive $CD4^+$ T cell pool of healthy individuals decreased from ~80% in neonates to 60% around the age of 30, and 40% at the age of 60, our TREC analyses pointed out that, throughout adulthood, maximally 30%—and on average only 11%—of the naive T cell pool was originally formed by the thymus. In line with this, we found that in adults, as many as 77% of $CD31^+$ naive $CD4^+$ T cells were in fact formed by peripheral T cell proliferation. Although these data show that in terms of naive T cell numbers created per day, peripheral T cell proliferation by far exceeds thymic output in human adults, the thymus may still have an essential role – if only because new T cell specificities can only be created by the thymus.

In contrast to the large contribution of peripheral T cell proliferation to the maintenance of naive T cells in humans, we here show that naive T cells in euthymic mice are almost exclusively

formed by thymic output. Such a limited role for peripheral naive T cell division in mice is fully compatible with in vivo BrdU-labeling results in several classical studies (Tough and Sprent, 1994; von Boehmer and Hafen, 1993), which showed that BrdU accrual in naive T cells of young adult mice strongly depended on the presence of a thymus. Here, we extend this observation by showing that naive T cell division hardly occurs not only in young-adult mice but even in mice in old age. Apparently, if present at all, most naive peripheral T cell proliferation in mice induces loss of the naive phenotype. This agrees well with the view that most naive T cells triggered to divide in lymphopenic mice obtain effector or memory (like) T cell characteristics (Surh and Sprent, 2008). The conclusion that peripheral T cell proliferation hardly contributes to the maintenance of the naive T cell pool in euthymic mice was independently confirmed by the lack of TREC dilution in the naive T cell compartment of aging mice. In contrast, the average naive T cell TREC content in humans has been shown to decline by 90% to 99% (Harris et al., 2005; Jamieson et al., 1999; Kilpatrick et al., 2008). Although one could argue that mice do not live long enough for changes in naive T cell TREC contents to occur during their life, our quantification of T cell turnover in mice (this paper) and men (Vrisekoop et al., 2008) has demonstrated that naive T cell turnover rates in mice are ~40-fold higher than in humans. If naive T cell production in mice were to be due to peripheral T cell proliferation, as it is in humans, the decline of naive T cell TREC contents over 2 years in mice would thus be expected to be similar to the decline observed in aging humans.

The interpretation of TREC data remains difficult because the average TREC content of the naive T cell pool reflects a complex balance between thymic output, T cell proliferation, and cell death (De Boer, 2006; Hazenberg et al., 2000; Ribeiro and de Boer, 2008). We have shown that the normalized TREC content (defined by Equation 1) nevertheless quantifies which fraction of all naive T cells that are produced per day is generated by the thymus. In this equation we have assumed that TRECs themselves do not decay, unless their host cell dies. It is indeed widely assumed that TRECs are extremely stable, in part because TRECs have been shown to persist for decades in fully thymectomized individuals (Douek et al., 1998; Halnon et al., 2005; Prelog et al., 2009; Sempowski et al., 2001). Because a role for TREC decay cannot formally be ruled out, however, we sought for further—TREC-independent—arguments that support our claim that naive T cell maintenance is different in mice and men.

The first argument comes from estimates of thymic T cell production in mice and men. Studies in different species have shown that the thymus exports ~1% of thymocytes per day (Binns et al., 1988; Holder et al., 2006; Scollay et al., 1986). A human thymus has been shown to contain on average $\sim 10^7$ thymocytes per gram of thymic tissue at the age of 20 years, which further declines with age (Marusić et al., 1998), and the total weight of the human thymus in adults between 20 and 84 years of age has been shown to be on average 23 g and to be remarkably constant with age (Steinmann et al., 1985). With a daily export rate of 1%, naive T cell export from the thymus would thus be estimated to be maximally 2.3×10^6 cells per day. Our heavy-water studies show that total daily naive T cell production in humans is much higher, in the order of 10^8 naive T cells per day (calculated as $p \times$ [the naive cell count per liter blood] $\times$ 5 l blood $\times$ 50, assuming that 2% of lymphocytes reside in the blood) (Vrisekoop et al., 2008). If all these new naive T cells in humans were to be produced by the thymus, it would mean that—in contrast to all other species investigated—in humans as much as 44% of thymocytes would have to be exported into the periphery on a daily basis. A much more likely explanation is that the vast majority of the 10^8 naive T cells that are produced per day in a healthy human are produced by peripheral T cell proliferation. In mice, in contrast, the total production of 4×10^5 naive T cells per day in the spleen that we measured using heavy-water labeling is perfectly in line with a ~1% output of thymocytes per day. Because the number of naive T lymphocytes in the spleen represents ~30% to 40% of the total number of naive T lymphocytes in the mouse, and our deuterium-labeling studies showed that T cells in lymph nodes and spleen behave kinetically similarly (data not shown), the total daily production in a young adult mouse is ~1.0 to 1.3×10^6 naive T cells. All these cells can be produced by the thymus given that the thymus of a young adult male mouse contains $\sim 1.2 \times 10^8$ thymocytes (data not shown).

The second argument is based on the quantification of total daily T cell production versus daily T cell proliferation. Our deuterium-labeling results in mice and men point out that the fraction of the naive T cell pool that is being renewed per day differs dramatically between mice and men: In mice, 2.1% and 1.3% of the naive $CD4^+$ and $CD8^+$ T cell pool is being replaced per day, whereas in humans, as little as 0.05% and 0.03% of the naive $CD4^+$ and $CD8^+$ T cell pool is replaced per day; i.e., in mice, naive T cells are renewed ~40 times more quickly than in men. If the relative contribution of thymus output and peripheral T cell proliferation were to be similar in mice and men, one would predict that T cell proliferation rates in men should be ~40 times lower than in mice. When we measured the expression of the intracellular proliferation marker Ki67 of naive T cells in both mice and men—on the basis of the very same markers that were used to distinguish naive T cells in our deuterium-labeling studies—we found, however, that the fraction of the naive T cell pool that is proliferating at any moment in time is very similar between mice and men (data not shown). Together, these data show that the relative contribution of peripheral proliferation to the maintenance of the naive T cell pool must be completely different in mice and men.

The finding that naive T cells in mice have a relatively short life span, and are almost completely thymus derived, contrasts strongly with the long life span of naive T cells and the predominant role of peripheral T cell proliferation in the maintenance of the naive T cell pool in humans. We defined the human naive T cell population by the expression of CD27 and lack of CD4RO expression (Baars et al., 1995; Hamann et al., 1997). It is important to stress that the human naive T cells that we thus analyzed were naive according to all commonly accepted phenotypical markers for naive T cells (including CD62L, CCR7, CD127, and CD28; data not shown). Conversely, the expression of CD95 and CD57, which is known to be very low on naive T cells, was indeed very low within our $CD45RO^-CD27^+$ (naive) T cell populations (data not shown). We thus conclude that by all current standards we have studied what is conventionally defined as naive T cells in humans. Our

TREC data of CD31$^+$ naive T cells provide further support for the fundamental difference in naive T cell maintenance between mice and men. CD31 is generally thought to be a marker for naive T cells that are most proximal to the thymus (Kilpatrick et al., 2008; Kimmig et al., 2002; Kohler et al., 2005). The observation that even the majority of CD31$^+$ human naive T cells have been produced by peripheral T cell proliferation demonstrates that our results are not due to the specific markers that we used.

These results not only have bearing on our ideas about T cell renewal in healthy individuals with a full T cell compartment but are also critical to our understanding of human T cell reconstitution in clinical conditions characterized by severe T cell depletion. The quantification of naive T cell production by the thymus and by peripheral proliferation demonstrates that the contribution of the thymus in young healthy adults is much smaller than widely assumed on the basis of the mouse model. Indeed, the major implication of this work is that one cannot freely extrapolate insights about naive T cell kinetics in young adult (or old) mice to young adult (or old) humans, or vice versa, for the source by which naive T cells are produced differs qualitatively between mice and men.

EXPERIMENTAL PROCEDURES

Blood and Tissue Samples

Buffy coats or whole heparine anticoagulated blood samples were obtained by venapuncture from human blood bank donors and children visiting the UMCU. Cord blood samples were obtained from healthy full-term neonates directly after delivery. Thymocytes were obtained from children who were thymectomized because of complicated heart surgery at very young age. Written informed consent was obtained from all participants or their legal guardians in agreement with the Helsinki Declaration of 1975, revised in 1983.

Mice

C57Bl/6 mice were maintained by in house breeding at the Netherlands Cancer Institute in Amsterdam or the Central Animal Facility at Utrecht University under specific pathogen-free conditions in accordance with institutional and national guidelines. Thymectomy was performed at 7 weeks of age as described before (Vrisekoop et al., 2008). Completeness of thymectomy was confirmed by visual inspection, both directly after removal of the organ and at the end of the experiment. Only fully thymectomized animals were included in this study.

Cell Preparation and Flow Cytometry

Blood mononuclear cells were obtained by Ficoll Paque density gradient centrifugation and cryopreserved until further use. Single-cell suspensions were obtained by mechanically disrupting spleen, thymus, or (axillary, brachial, inguinal and superficial cervical) peripheral lymph nodes (PLNs). Red blood cells in blood samples were lysed with ammonium chloride solution (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA [pH 7.4]). For measuring the fraction of CD31$^+$ T cells within the human naive CD4$^+$ T cell population and for purifying CD4$^+$CD45RO$^-$ CD27$^+$CD31$^+$ and CD4$^+$CD45RO$^-$ CD27$^+$CD31$^-$ cells, cryopreserved PBMC were thawed and incubated with monoclonal antibodies (mAb) to CD45RO (Caltag), CD31, CD4 (BD), and CD27 (Sanquin Reagents), after which they were analyzed (using Cellquest or FACS Diva software) or sorted on a flow cytometer (FACS Calibur, LSRII, FacsAria [BD] or MoFlow [Dako]). In 13 of 18 sorts we omitted the mAb to CD27 but confirmed that the percentage contaminating effector CD4$^+$ CD45RO$^-$CD27$^-$ T cells was on average only 0.75%. Sort purity was on average 93%. For determining the fraction of the different mouse T cell populations, fresh cells were washed, resuspended in IMDM/7% FCS, and counted. For isolating naive (CD62L$^+$, CD44$^{-/lo}$) and effector-memory (CD62L$^+$, CD44^{hi} and CD62L$^-$CD44^{hi}) CD4$^+$ and CD8$^+$ T cells, splenocytes and LN suspensions were stained with CD4 (clone RM4-5) or CD8 (clone 53-6.7), in combination with CD44 (clone IM7) and CD62L (clone MEL-14) (BD) in the presence of blocking 2.4G2 mAb in PBS/1% BSA. Sort purity was 98.4% ± 1.0% (naive CD4$^+$ T cells), 97.2% ± 1.6% (effector-memory CD4$^+$ T cells), 98.4% ± 1.1% (naive CD8$^+$ T cells), and 96.0% ± 2.1% (effector-memory CD8$^+$ T cells). To isolate single positive CD4$^+$CD8$^-$ thymocytes, thymocyte suspensions were stained with anti CD4 and CD8 (BD) and sorted by flow cytometry. Mouse thymocytes were also stained for CD44 (BD) to exclude peripheral T cells that reentered the thymus (CD44^{hi}). Sort purities were 93.5% ± 3.3% (human CD4$^+$ SP), 97.1% ± 1.9% (mouse CD4$^+$ SP) and 90.5% ± 3.4% (mouse CD8$^+$ SP). Isolated mouse cells were frozen until further processed.

TREC Analyses

Genomic DNA was isolated with the QIAamp Blood Kit in accordance with the manufacturer's instructions (QIAGEN, Hilden, Germany). Mouse genomic DNA was isolated by cell lysis (10,000 cells/μl) in 0.05% Tween-20, 0.05% NP-40, and 100 μg/ml Proteinase K for 30 min at 56°C and then for 15 min at 99°C. Lysates were spun down for 15 min. Signal joint (Sj) TREC numbers and DNA input were quantified with an ABI Prism 7900HT Real Time PCR System (Applied Biosystems, Foster City, CA). The number of human Sj TREC copies in a cell population and the input DNA were determined and calculated as described previously (Hazenberg et al., 2000). For the TREC content within naive CD4$^+$ T cells, we combined previously measured healthy controls (TREC content within CD4$^+$CD45RA$^+$) (Hazenberg et al., 2000) with data from 11 healthy volunteers (TREC content within CD4$^+$CD45RO$^-$CD27$^+$, average sort purity of 98%), including four volunteers from a ^2H_2O-labeling study (Vrisekoop et al., 2008). For 17 individuals the TREC content of naive CD4$^+$ T cells was calculated with the formula: (fraction CD31$^+$ within naive CD4$^+$ T cells) × (TREC content CD31$^+$ naive CD4$^+$ T cells) + (1 − fraction CD31$^+$ within naive CD4$^+$ T cells) × (TREC content CD31$^-$ naive CD4$^+$ T cells). Mouse TREC analysis was performed as described (Broers et al., 2002). To correct for input genomic DNA, we separately amplified the CD45 reference gene by forward primer 5′-TCAGAGGCCAGGCTCACTCAAG-3′ and reverse primer 5′-CTAGGCCAACCACTCCCACTGT-3′ (MWG Operon, Ebersberg, Germany) and detected it by fluorescent probe FAM-5′-CAATGTT CAAGTTGCCCAGCGATGCCAGC-3′-TAMRA (Applied Biosystems, Foster City, CA).

^2H_2O Labeling

^2H_2O labeling was achieved by giving mice one boost injection (i.p.) of 15 ml/kg phosphate buffered saline (0.9% NaCl w/v in 99.8% ^2H_2O [Cambridge Isotopes, Cambridge, MA]) and subsequently feeding them with 4% ^2H_2O in the drinking water for 4 weeks.

Measurement of ^2H_2O Enrichment in Plasma and DNA

Deuterium enrichment in plasma was measured as reported by Previs et al. (1996). The isotopic enrichment of DNA was determined as previously described (Vrisekoop et al., 2008).

Mathematical Modeling of ^2H_2O Data

Enrichment data were fitted with a previously developed mathematical model (Vrisekoop et al., 2008), which was extended to take into account a delay with which labeled cells reach the spleen (Pillay et al., 2010). Best fits were determined by minimizing the sum of squared residuals after arcsin (sqrt) transformation. Availability of deuterium was measured in plasma, and all cellular enrichment data were normalized by the maximal enrichment level observed in thymocytes (Vrisekoop et al., 2008).

Mathematical Modeling of Thymectomy Data

We devised a mathematical model to quantify naive T cell dynamics in control and thymectomized mice, assuming that under normal conditions, naive T cells are produced by thymic output and peripheral T cell proliferation and lost via differentiation into effector-memory T cells and cell death. Thymic output was described by a phenomenological function $f_1(t)$, which was fitted to the number of SP thymocytes (data not shown) and multiplied by ε, the fraction of SP thymocytes exported to the spleen per day. The function is

a modification of the thymus involution function described by Steinmann et al. (1985) and is explained below.

In the model, both cell death rates (d_nN) and proliferation rates ($r/(1+N/h)$) could be density dependent; i.e., cells live longer and/or proliferate more frequently under lymphopenic conditions, when survival or stimulatory signals are abundant. The differential equation for the number of naive T cells (N) is:

$$\frac{dN}{dt} = \varepsilon f_1(t) + \frac{r}{1+N/h}N - d_nN^2$$

We considered two extreme cases of this model by allowing for:

(1) density-independent proliferation and density-dependent death rates, i.e.:

$$\frac{dN}{dt} = \varepsilon f_1(t) + rN - d_nN^2,$$

or for:

(2) density-independent death and density-dependent proliferation rates, i.e.:

$$\frac{dN}{dt} = \varepsilon f_1(t) + \frac{r}{1+N/h}N - d_nN.$$

Because naive T cells have an intrinsic (limited) lifespan (Di Rosa et al., 1999), a density-independent death term should always be present. In model (1), proliferation, differentiation, and density-independent cell death rates were all combined in a single net proliferation rate r. In model (2) the net death rate d_n includes both cell death and differentiation.

Because T cells continuously recirculate through the body, we assumed that naive T cell dynamic parameters are equal in different organs. We therefore simultaneously fitted the dynamics in the spleen and PLN and related the two with a proportionality function, $f_2(t)$, described below. The parameters of the function $f_2(t)$ were first determined by fitting the function to all cell numbers available from the two organs of euthymic and thymectomized mice (data not shown), i.e., $N_{PLN} = f_2(t)\,N_{Spleen}$, where N_{PLN} and N_{Spleen} are the numbers of naive $CD4^+$ and $CD8^+$ T cells in peripheral lymph nodes and spleen, respectively. Parameter estimates of the differential equations were obtained by fitting the total cell number, N, to the data (taking the natural logarithm) based on the Levenberg Marquardt algorithm (Marquardt, 1963) for solving nonlinear least-squares problems.

Mathematical Modeling of the Thymic Output and Proportionality Functions

We described both the thymic output function and the proportionality function between cell numbers in lymph nodes and spleen by a phenomenological function $f_i(t)$ (where $i = 1$ for the function describing thymic output and $i = 2$ for the proportionality function):

$$f_i(t) = \begin{cases} \sigma(1-e^{-s_1 t}); & t \le T_{off} \\ f_i(T_{off})\left[\alpha e^{-s_2(t-T_{off})} + (1-\alpha)e^{-s_3(t-T_{off})}\right]; & t > T_{off} \end{cases}$$

The function is composed of a sum of exponents with constants s_1, s_2, and s_3; the two exponental decays for $t > T_{off}$ are weighted by a constant $0 \le \alpha \le 1$.

Statistical Analysis

Normality of the human data was tested with the Shapiro Wilk W test for normality. On the basis of the outcome of this test, we calculated correlations with Pearson's (R_p) or Spearman's rank correlation coefficients (R_s). The Mann-Whitney U test was used for determining differences between group characteristics. Differences between the TREC content within $CD31^+$ and $CD31^-$ naive $CD4^+$ T cells were analyzed with the Wilcoxon signed-rank test. We tested whether there was a significant difference between the rate of TREC loss in $CD31^+$ and $CD31^-$ naive T cells with a linear model including an interaction term between age and group ($CD31^+$ versus $CD31^-$). Statistical analyses were performed with SPSS 15.0 (SPSS, Chicago, Illinois). Differences with $p < 0.05$ were considered significant. Mathematical models for mouse cell densities (model 1 and model 2) were compared on the basis of sums of squared residuals. The 95% confidence intervals (CIs) for the inferred parameters were determined with a bootstrap method (Efron and Tibshirani, 1986), in which the residuals to the optimal fit were resampled 500 times.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and two tables and can be found with this article online at doi:10.1016/j.immuni.2012.02.006.

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